Studies on the Carotenoid and Glycosidic Precursors of Aroma and Flavour compounds in *Boronia megastigma* (Nees)

by

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Chris Cooper
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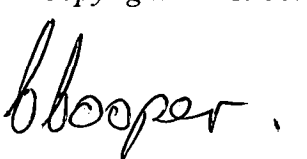

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Abbreviations:

APCI	- Atmospheric Pressure Chemical Ionisation
ATP	- Adenosine triphosphate
C18	- Octadecane (used as an internal standard in Gas Chromatography)
C- No.	- The carbon length of molecules is referred to in the text as C followed by a number. Eg C-27 refers to a 27 carbon chain length molecule such as a C-27 apocarotenoid
CCD	- Carotenoid Cleavage Dioxygenase
DCM	- Dichloromethane
DCCC	- Droplet Counter Current Chromatography
FID	- Flame Ionisation Detector
GC	- Gas Chromatography
GC/O	- Gas Chromatography / Olfactometry
HP	- Hewlett Packard
HPLC	- High Performance Liquid Chromatography
HRGC	- High Resolution Gas Chromatography
i. d.	- Internal Diameter
LC	- Liquid Chromatography
MS	- Mass Spectrometry
MEP	- Methylerythritol Phosphate
MLCCC	- Mulit Layer Coil Counter Current Chromatography
PDMS	- Polydimethylsiloxane
psi	- Pounds per Square Inch
PVPP	- Polyvinylpolypyrrolidone
RLCC	- Rotation Locular Counter-current Chromatography
RP C-18	- Reversed Phase C-18 resin for HPLC
RT	- Retention Time
SDE	- Simultaneous Distillation Extraction
SPME	- Solid Phase Micro-Extraction
TIC	- Total Ion Current
XAD-2	- Polystyrene resin used for the adsorption of glycoconjugates

Definitions:

Absolute: The ethanol soluble portion of a concrete that may also be decolourised.
Concrete: A concrete is the primary solvent extract of a plant or plant part using a non polar solvent.
Glycosidic conjugates: Glycoside is both a general term applied to all forms of glycosidic conjugates and in this thesis to the simplest form of hexyl sugar conjugates. Hence in order to differentiate between the generic meaning of the term, malonyl glycosides, and hexyl mono-carbohydrate glycosides the later are referred to in the text as simple glycosides.
Marc: The waste plant material obtained following processing which in the case of boronia refers to residual flowers after extraction with petroleum ether.
Pottle: A small autoclavable plastic jar used for growing cultures in various media.

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Abstract

Studies relating to the carotenoid precursors of C-13 norisoprenoids resulted in the identification of five C-27 apocarotenoids. The new compounds were 3-hydroxy-apo-10′-carotenoic acid, methyl 3-hydroxy-apo-10′-carotenoate, apo-10′-carotenoic acid, apo-10′-carotenal, and methyl apo-10′-carotenoate. This provided evidence for site specific cleavage of C-40 hydrocarbon carotenoids and xanthophylls in the 9, 10 position. Changes in the levels of these apocarotenoids, β-ionone and C-40 carotenoids demonstrated that metabolism relating to carotenoid cleavage increased during flower opening.

Glycosides of monoterpenes, C-13 norisoprenoids and cucurbates were detected in boronia using an HPLC screening process followed by enzymatic hydrolysis of an extract using both a pectinase and B-D-glucosidase. Release of these conjugates was further demonstrated through a simple marc incubation process. The pattern of change over a ten day period demonstrated initial release of the compounds over 4 – 6 days of incubation followed by their disappearance. A commercial trial of the process demonstrated a 36% increase in the yield of extract after 4 days. GC / Olfactometry indicated that many of the compounds in the new extract had aroma properties compatible with the primary boronia extract. There was additionally evidence that tiglamides, cinnamate esters and 8-hydroxy linalool esters that were previously not considered as flavour and aroma compounds contributed positive aroma sensations.

Endogenous fungi were isolated from boronia which have the capacity to breakdown glycosides of flavour and aroma compounds including monoterpenols, C-13 norisoprenoids and cucurbates. These fungi were tested in a model system using sterilised boronia marc. The impact of fungal treatment on 45 naturally occurring boronia compounds was measured over a 4 day incubation period. The results indicated a complex pattern of changes including hydrolysis of glycosides, further metabolism of released compounds, and metabolism of volatile compounds remaining after the primary flower extraction.

Chapter 1

Literature Review

1.1 Background

Boronia megastigma (Nees) (Brown Boronia, family *Rutaceae*) is a woody understorey shrub that is endemic to the South-West of Western Australia. The plant produces a highly fragrant flower that is deep red-brown on the outside with an intense yellow colouration on the inside. Fully open the flowers are up to 1 cm in diameter. An absolute from boronia flowers has been produced commercially from a petroleum ether extract since the 1920's. Guenther (1949), writing retrospectively, stated that early studies had indicated that steam distillation gave 'a very different' (and hence assumedly lower quality) product. The early extraction process involved hand harvesting in the wild with combs and transference of the flowers to drums of petroleum ether at field stations, before transportation to the factory for processing (Guenther, 1949). By 1928, boronia concrete was regarded as 'a well known article of commerce' (Ghisalberti, E. L., 1998).

New technologies were developed in the 1980's that established an industry in Tasmania. These technologies, solely owned by "Essential Oils of Tasmania" relate to the selection of high yielding clones, mechanical harvesting and the optimisation of petroleum ether extraction protocols. Currently in Tasmania, four clones of *Boronia megastigma* (Nees) are grown commercially to produce an absolute from a waxy concrete by extraction with ethanol and filtration. The absolute is currently used in the food industry to enhance berry fruit flavours in ice-cream, soft drinks and yoghurts. Although the absolute has excellent potential for perfumery because of the unique combination of β-ionone and methyl jasmonates (Mookherjee *et al.*, 1995), it has not been widely used for that purpose.

MacTavish (1995) made substantial progress during the 1990's in developing protocols for harvesting and post harvest processes that gave increases in the yield of volatiles. Much of the focus of these experiments was on β-ionone production due to

the exceptional flavour and aroma value of this compound (Arctander, 1960) and its low flavour threshold (Tan and Siebert, 2004). MacTavish and Menary (1997) found that both the extract as a percentage of fresh flower weight and the concentration of β-ionone increased up until flower opening reached 70% in a crop. A further study, involving separate examination of the four different commercially produced clones of boronia (3, 5, 17, 250), resulted in more detailed recommendations for field harvesting of different clones in the range 75% - 90 % of open flowers (MacTavish and Menary, 1999a). These figures intrinsically imply that β-ionone levels in flowers begin to decrease after flower opening.

Subsequent work by these authors found that post harvest incubation at between 12 and 25°C for up to 24 hours resulted in increased levels of extract of up to 25% with higher percentage increases in some volatiles including β-ionone (MacTavish and Menary, 1998a). This post harvest effect was examined with regard to clonal type and incubation temperature (MacTavish and Menary, 1999b), and oxygen consumption (MacTavish and Menary, 1999c). The selective use of respiratory inhibitors in this later study indicated that these post harvest changes were dependent on active cellular respiration, as measured through oxygen consumption in whole flower studies, which did not require ATP. However the mechanism for post harvest increases in volatiles is not known. Possible explanations include:

(1) Fungal metabolism of existing carotenoids to yield C-13 norisoprenoids. Related to that it was observed (personal communication, Menary, R.) in some boronia clones that fungal activity occurs on flowers before harvest.
(2) Release of volatiles from cellular compartments due to post harvest break-down in the cellular structure.
(3) Continuing biosynthesis of secondary metabolites.

The laboratory observations with regard to post harvest increases in volatile production were examined at a pilot scale and a methodology developed with on-farm application without temperature control (MacTavish and Menary, 2000).

Additionally, MacTavish *et al.* (2002) reported the release of volatiles, including β-ionone, following incubation with endogenous enzyme fractions. However, the increase in these volatiles was very low (personal communication, Davies, 2002) and

several compounds not able to form glycosidic bonds were found to increase in the experimental system. Although considered a possibility due to these observations, the large increases in β-ionone that resulted from the post harvest incubation described above is thought to be unlikely to result from the hydrolysis of glycosides because β-ionone does not contain a free hydroxyl group and a two step process would be necessary. A consideration of boronia volatile compounds that could possibly form glycosidic conjugates will be considered in another section of this review.

It is important to note that MacTavish and Menary (1999c) observed that large buds and flowers consumed up to three times as much oxygen in post harvest incubations than was consumed by smaller buds. The authors suggested this may imply a rapid increase in metabolism as buds mature which is a normal developmental pathway. The measurement of precursor changes during this period may provide valuable insights into both the existing metabolic process and the potential for increasing yields from carotenoid and glycosidic precursors.

Additional advances include observations that other post harvest mechanisms also resulted in increased volatile (including β-ionone) production. The rolling and pressing of fresh flowers prior to extraction resulted in a 48% increase in volatiles with a 23% increase in β-ionone (MacTavish and Menary, 1998b). This translated into a 127% increase in the yield of absolute and the authors suggested that the process may have enhanced the extraction of polar compounds. The authors suggested that increased diffusion processes enhance the yield of extract by breaking down the cellular structure. They refer to a study by Georgiev and Balinova-Tsvetkova, (1977) where it is commented that petroleum ether does not penetrate well into the wet tissue of plants. In the later study this is used to explain the lower yields obtained in the extraction of lavender with petroleum ether compared to the use of more polar solvents.

Physico-chemical processes, including solvent penetration and partitioning effects, are clearly an important aspect for consideration when discussing possible reasons for yield improvements. However, MacTavish and Menary (1998b) overlook the

potential contribution of metabolic processes. With regard to that the breakdown in cellular compartments may lead to enzymatic reactions such as lipoxygenase activity (Fitz *et al.*, 1999). This activity has been demonstrated to lead to increases in the yield of both off-flavours and beneficial flavour compounds such as β-ionone through auto-oxidation of un-saturated fatty acids and carotenoid co-oxidation processes respectively (Fitz *et al.*, 1999). The implications of this for boronia have not been explored but it is clear that carotenoids may be potential flavour and aroma precursors in boronia.

Whilst advances in these boronia technologies (MacTavish, 1995; MacTavish and Menary, 1999b; MacTavish and Menary, 1999c; MacTavish and Menary, 2000) have resulted in increased yields from fresh flowers it is timely to review the potential to obtain even further increases of yield. Overall, the increases in the yield of boronia and β-ionone from post harvest processes imply the possibility that a range of mechanisms exist in boronia that have led to these improvements. However, there is insufficient knowledge of the relevant mechanisms and processes and the potential may exist for increases in the yield of other important volatiles. A consideration of metabolic and biotechnological processes with regard to potential carotenoid and glycosidic precursors may provide important information. Consequently this literature review will consider four main lines of investigation:

1. A consideration of carotenoid occurrence, isolation and metabolism in relation to the role of these compounds as precursors to ionone compounds which are key flavour ingredients in boronia.
2. Knowledge of glycosides and the biotechnological processes for release of bound flavour and aroma compounds.
3. The current state of knowledge with regard to the volatile composition of boronia extract with a critical assessment of the relationship of these compounds to plant metabolic process that may play a role in providing a pool of flavour and aroma precursors.
4. The aroma characteristics of boronia volatiles and the potential for compounds that may be released from precursors to contribute to the extract's yield and quality.

The final section of the review will consider potential outcomes and the experimental approach to the subject.

1.2 Carotenoids

1.2.1 General Background

The widespread occurrence of carotenoids in plants is recognised because of their impact on human health especially with regard to vision (Simpson and Chichester, 1981) and their role as antioxidants (Britton, 1995; Edge *et al.*, 1997). Carotenoids have complex roles in plants including for photoprotection during photosynthesis and as pollinator attractants through colouration of flowers (Bartley and Scolnik, 1995). These molecules are integrated into the thylakoid membranes of higher plant chloroplasts (Britton, 1998) but are generally not visible in the leaves until their yellow, red and orange hues are revealed through chlorophyll degradation (Matile, *et al.*, 1999) with spectacular effect in many deciduous plants.

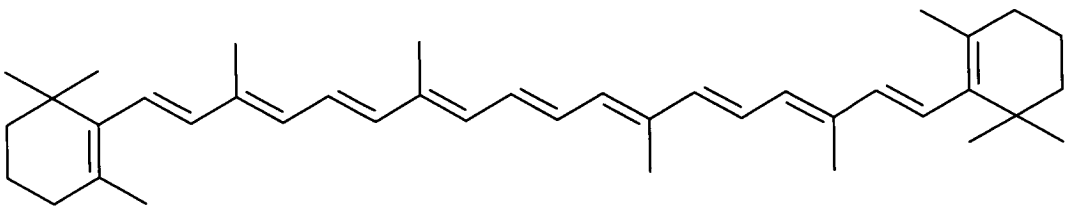
From a metabolic point of view, carotenoids belong to the isoprenoid family of compounds, which are variously estimated to include over 20,000 molecules (Eisenreich *et al.*, 2004; Holstein and Hohl, 2004). Isoprenoids are derived from isopentenyl diphosphate which is synthesised either via the mevalonate or the more recently discovered methylerythritol phosphate (MEP) pathways (Dewick, 2002, Kuzuyama, 2002; Eisenreich *et al.*, 2004). The review by Dewick (2002) claimed that the mevalonate pathway operates in the cytosol with a particular emphasis on the production of triterpenoids, steroids and some sesquiterpenoids. The MEP pathway operates in plastids for biosynthesis which includes monoterpenes, diterpenes, some sesquiterpenes and carotenoids. Biosynthesis of C-40 carotenoids occurs via the well-described pathway of head to tail condensation of isopentenyl diphosphate units to produce geranylgeranylpyrophosphate (GGPP) followed by head to head condensation of two GGPP units to produce phytoene (Goodwin, 1993; Britton, 1998). Subsequent stepwise desaturation of phytoene leads to lycopene which can undergo a number of biosynthetic reactions including cyclisation and hydroxylation to produce a variety of C-40 carotenoids (Britton 1998).

The many types of carotenoids are elegantly classified by Straub (1987). These include the C-40 hydrocarbon carotenoids such as β-carotene, and xanthophylls such

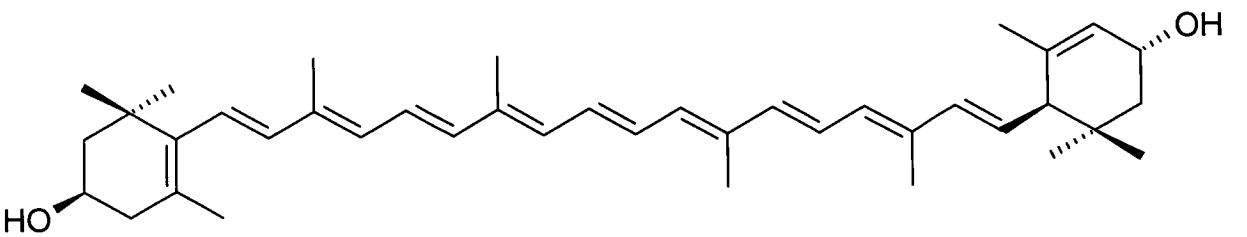
as zeaxanthin, and lutein. Also listed are a range of longer chain homocarotenoids and with particular importance to this study, the apocarotenoid cleavage products of the C-40 molecules. Progress with regard to the identification of new carotenoids has been reviewed by Kull and Pfander (1995) and Mercadante (1999a). Additionally, the *Carotenoids Handbook* (Britton *et al.*, 2003) updated the *Key to Carotenoids* (Straub, 1987) and provided an invaluable research tool for those interested in carotenoid identification.

The same four compounds are found as the main carotenoids in most plant leaves namely, β-carotene (20-30%), lutein (~45%), violaxanthin (~15%), and neoxanthin (~15%) (Britton, 1998). The structures of these carotenoids are detailed in **figure 1.1**. Structural consideration of these molecules and of other carotenoids found in flowers and fruit have been important with regard to examining their role as precursors of volatile compounds. C-40 carotenoids have long been thought to be precursors of volatile compounds ranging in carbon length from 9 to 13 carbon atoms (Enzell, 1985; Lutz and Winterhalter, 1992). The next section of the review will discuss the precursor role of carotenoids in detail.

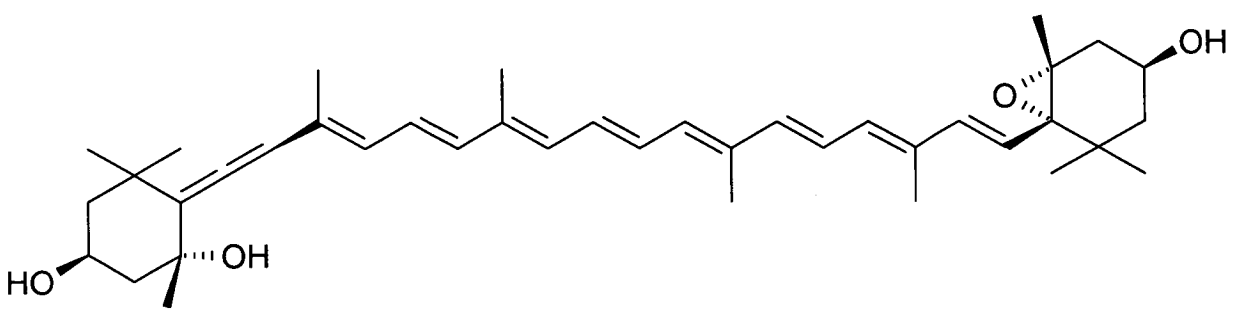
However, firstly it is important to briefly consider some issues with regard to the identification of carotenoids which may provide valuable insights with regard to the role of these compounds as precursors of flavour and aroma volatiles, especially including β-ionone and other C-13 norisoprenoids previously identified in boronia (Weyerstahl *et al.*, 1995). Although it is generally accepted that full elucidation of new molecular structures requires NMR spectroscopy, mass spectrometry has provided an important contributing tool for the structural elucidation of carotenoids over three decades (Enzell and Back, 1995). Furthermore the identification of carotenoids has been aided since the mid 1990's by the availability of HPLC-MS methodology, which allows fast separation of carotenoids, with tandem UV-Vis (diode-array) and mass spectrometry (van Breeman *et al.*, 1996; van Breeman, 1997; Müller, 1997, Herderich. 2002; Cortés *et al.*, 2004). This methodology combined with the use of standards is now used routinely for the quantitative analysis of plant carotenoids (Müller, 1997; Oliver and Palou, 2000).



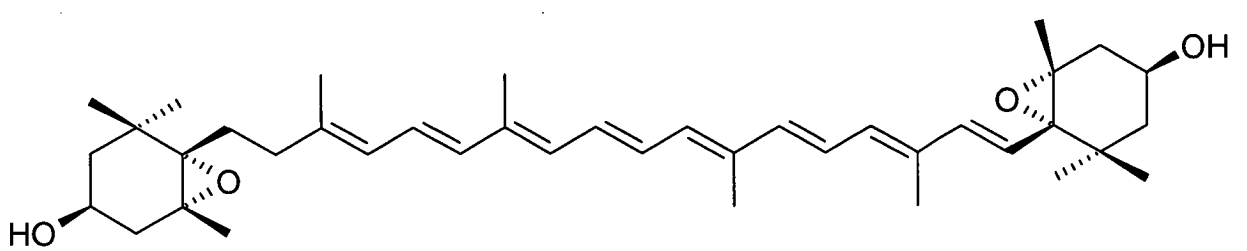
β-Carotene



Lutein



Neoxanthin



Violaxanthin

Figure 1.1 Structures of plant leaf carotenoids.

1.2.2 Bio-generation of C-13 Norisoprenoids from C-40 Carotenoids

Arguments supporting an apocarotenoid pathway for the formation of C-13 norisoprenoid aroma compounds from C-40 carotenoids have been discussed in the literature. This evidence included the structural matching of apocarotenoid fragments (including relevant stereochemistry) with the proposed C-40 carotenoid parent molecules and the finding that norisoprenoid levels increased whilst C-40 carotenoids decreased during some curing, fermentation and ripening processes (Knapp *et al.*, 2002). The evidence for matching end group fragments included an observation by Stevens (1970) that there was a high correlation between tomato volatiles and structurally related carotenoids in tomato. An early review (Enzell, 1985) described possible pathways leading to norisoprenoids varying from 13 to 9 carbon atoms in tobacco. In quince, Lutz and Winterhalter (1992) theorised that the finding of C-15 terpenoids, when considered along with previously discovered C13-norisoprenoid and matching C-10 and C-12 fragments from the central part of the polyene chain, was evidence of biosynthesis from C-40 carotenoids via non-specific cleavage (see **figure 1.2**).

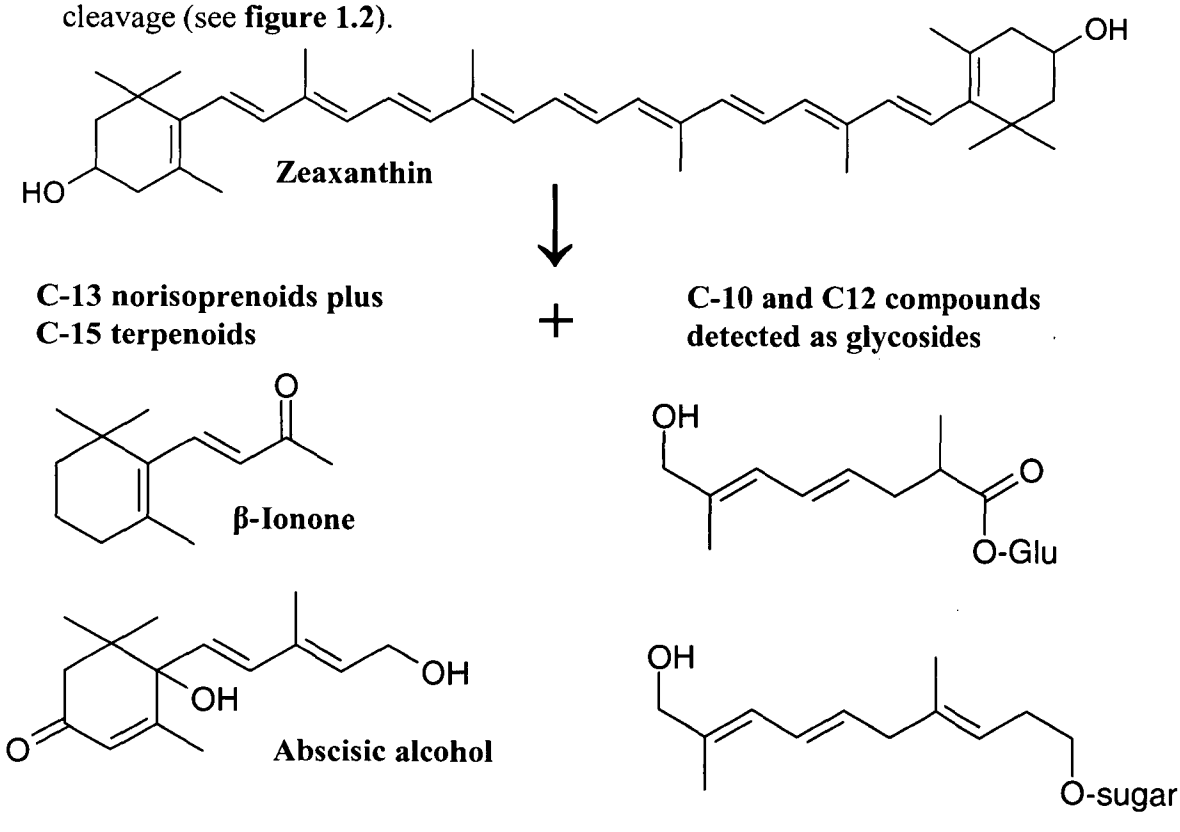


Figure 1.2 Hypothesised non-specific cleavage of quince carotenoids in the 9,10 and 11,12 positions to yield structurally related compounds.

In starfruit (Winterhalter and Schreier, 1995) a range of C-13 norisoprenoids were found to structurally match the end groups of C-40 carotenoid precursors. Additionally the finding of C-15 and C-10 molecules, matching carotenoid end groups and the central chain respectively, was again seen as evidence of volatile derivation from carotenoids. Similarly in saffron, crocetin (C-20) and a variety of C-10 molecules including the important flavour compound safranal, had been postulated to be derived from the C-40 molecule zeaxanthin (Winterhalter and Straubinger, 2000; Cadwallader, 2002). Extracts of rose flowers are known to contain, as does boronia, a wide range of C-13 norisoprenoids. Eugster and Marki-Fischer (1991), based on the identification of C-27 apocarotenoids and rosafluene (C-14), postulated a two step biosynthetic process in roses as outlined in **figure 1.3**. The first step proposed was cleavage in the 9,10 position to form one C-13 norisoprenoid molecule and a C-27 apo-carotenoid. This later molecule is further cleaved to form the C-14 rosafluene and a second C-13 norisoprenoid molecule.

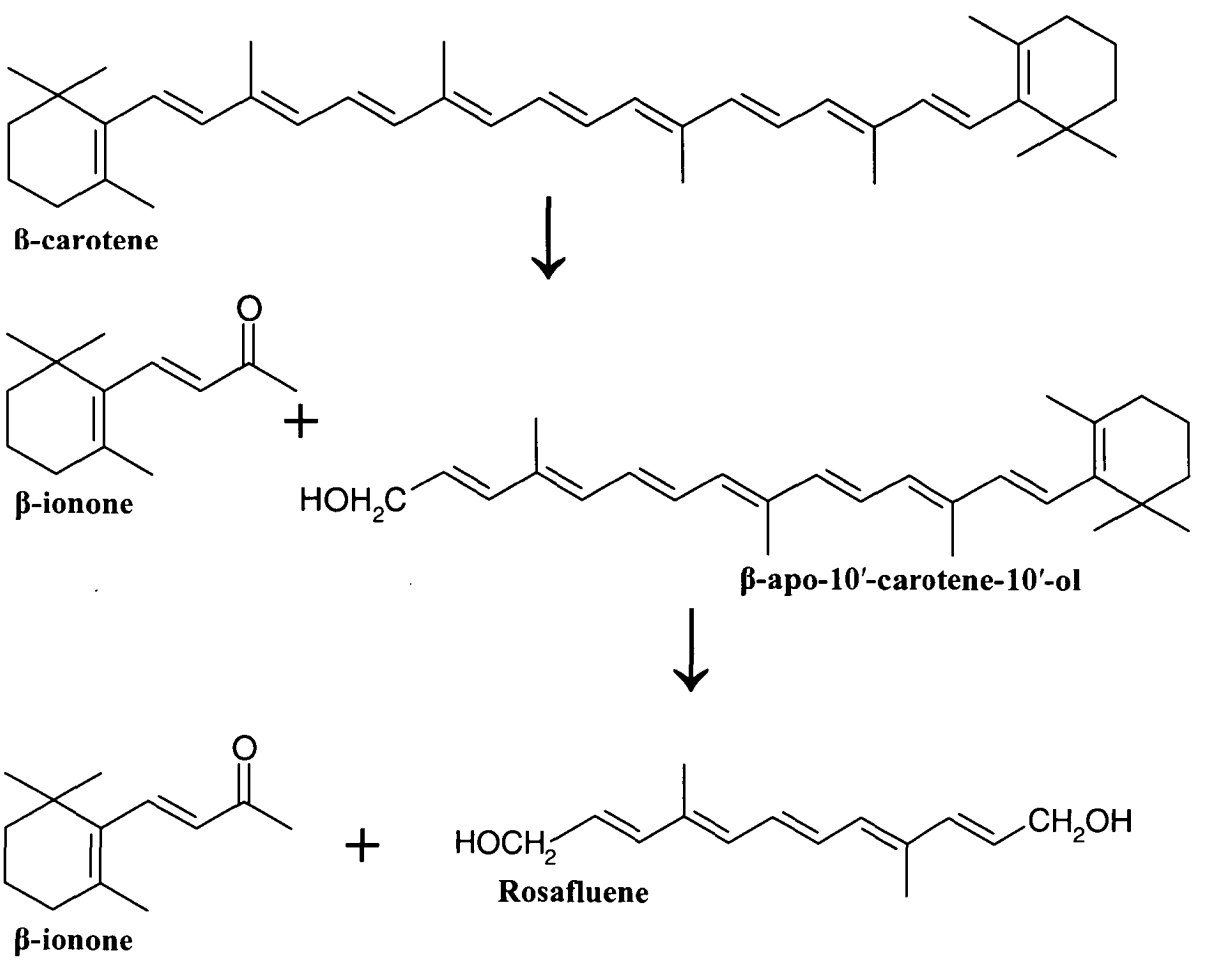


Figure 1.3 Carotenoid cleavage yielding products with aroma and flavour value as proposed for rose flowers (Eugster and Marki-Fischer, 1991)

Until very recently there was little direct evidence for the presence in plants of an enzyme that cleaved C-40 carotenoids to yield norisoprenoids (Wahlberg and Eklund, 1998; Winterhalter and Rouseff, 2002). In animals the cleavage of ß-carotene to retinal is a physiologically essential biotransformation which can occur via centric (β,β-carotene-15,15'-monoxygenase) or excentric cleavage in the 9,10 position. (Wyss, 2004). Norcarotenoids were found in Microcystis cultures (Juttner, 1988) and a membrane bound carotene oxygenase, assigned as ß-carotene 7,8(7',8')-oxygenase was described (Juttner and Hoflacher, 1985).

With regard to researching the suggested mechanism for the production of C-13 norisoprenoids in plant systems, various models have been examined. Sanderson and Gonzalez (1971) demonstrated the oxidation of ß-carotene to ß-ionone in tea using a polyphenol oxidase system. However, volatiles were only produced when the reaction mixture was heated to 95°C, a temperature likely to cause thermal degradation of carotenoids. Later, it was demonstrated that co-oxidation of carotenoids occurred in lipoxygenase enzyme systems (Grosch *et al.*, 1977). A proposed free radical mediated mechanism for this co-oxidation was detailed by Wu *et al.*, (1999). Buonaurio and Servili (1999) hypothesised that induction of a lipoxygenase in chloroplasts, from a hypersensitive reaction resulting from bacterial infection, led to increased α-ionone and β-ionone production in pepper leaves.

Furthermore, Biswal (1995) reviewed possible mechanisms for carotenoid breakdown during leaf senescence. Both free radical mediated mechanisms and the possibility of direct enzyme activity were discussed. This author also presented evidence that enzymes located in chloroplasts are implicated in carotenoid breakdown. Stratton *et al.* (1993) found that the product distribution from free radical producing systems depended on the nature of the free radical produced.

In the last 2-3 years however substantial progress was made with regard to the enzymatic cleavage of carotenoids, including the characterisation of carotenoid cleavage enzymes. Consequently, carotenoid cleavage in plants is now thought to be catalysed by a group of non-heme iron oxygenases (Giuliano *et al.*, 2003). The most widely researched example of a product formed through direct enzymatic cleavage

of carotenoids in plants is the plant hormone abscisic acid. Schwartz *et al.* (1997) demonstrated that a recombinant VP14 protein from a mutant maize was able to catalyse the cleavage of the epoxy-carotenoids violaxanthin and neoxanthin in the 11,12 position to xanthoxin, a precursor of abscisic acid. This gene has now been characterised in numerous plants including avocado (Chernys and Zeevaart, 2000), and Arabidopsis (Tan *et al.*, 2003) Additionally a *Bixa orellana* carotenoid cleavage enzyme has been expressed in *Escherichia coli*, cleaving lycopene to produce the classic colouring agent bixin (Bouvier *et al.*, 2003a). This is presumably through symmetrical cleavage in the 5,6 and 5',6' positions.

Research into VP-14 expression led to the discovery of a gene from *Arabidopsis thaliana* coding for an enzyme cleaving carotenoids in the 9,10 position (Schwartz *et al.*, 2001). Since then strong evidence, namely expression of the vector in bacteria and *in vivo* inhibition of the gene's expression in the tomato fruit, for a 9,10 cleaving CCD in tomatoes has been published (Simkin *et al.*, 2004). Bouvier *et al.* (2003b) have identified and characterised the existence of separate genes coding for cleavage of carotenoids in either the 7,8 or 9,10 positions. This is supported by the volatile profile in saffron of numerous C-10 and C-13 volatiles (Kanakis *et al.*, 2004).

Further work on the genetic expression of carotenoid cleavage enzymes by Tan *et al.* (2003) provided evidence that while the 9-cis epoxycarotenoid dioxygenase was located in chloroplast membranes, the carotenoid cleavage dioxygenase (CCD) is localised in the cytosol. The importance of understanding enzyme compartmentalisation especially with regard to the sequestration of carotenoid cleavage products in vacuoles, was briefly considered by Camara and Bouvier (2004). Certainly the finding that star fruit skin carotenase is localised in the cytosol (Fleischmann *et al.*, 2003) is in agreement with the discussion by the two previous authors.

Other investigators have characterised an enzyme cleaving carotenoids using classical biochemical techniques (non-gene based investigations). Fleischmann *et al.* (2003) clearly demonstrated cleavage of β-carotene in the 9,10 position through concurrent production of β-ionone in star fruit. Other studies with quince

(Fleischmann *et al.*, 2002) and nectarines (Baldermann *et al.*, 2005) whilst able to demonstrate β-carotene degradation by measuring a decrease of absorbance at 505 nm, did not demonstrate the concurrent appearance of norisoprenoids or apocarotenoids. The limitation in these studies was possibly due to the limited solubility (even in the presence of a detergent) of β-carotene in aqueous solutions. In this regard Fleischmann (2003) used a complex concentrating technique for the extraction of β-ionone prior to analysis.

In boronia, no direct evidence exists with regard to the mechanism of any carotenoid cleavage although MacTavish (1995) in an *in vitro* experiment was able to demonstrate that a protein extract from flowers caused a decrease in absorbance at 456.6 nm when incubated with β-carotene. However, the profusion and high concentration of C-13 norisoprenoids in boronia, which match the end-groups of known carotenoids, supports the rationale that a site specific process may be active in boronia. Importantly, no matching carotenoid cleavage products have been previously identified. Extensive identification of the volatiles in boronia (Davies and Menary, 1983; Weyerstahl *et al.*, 1994; Weyerstahl *et al.*, 1995) indicated the absence of rosafluene (or other C-14 volatiles) as is found in rose extracts. This absence might indicate that the parent carotenoid is cleaved only once and is not by itself evidence against the derivation of C-13 norisoprenoids from longer chain carotenoids. Should C-27 apocarotenoid fragments be found in boronia which matched the C-13 norisoprenoid compounds previously identified it would provide important evidence for enzymatic cleavage of boronia in the 9, 10 (9', 10') position.

1.2.3 A Biotechnological Perspective

It has been demonstrated that the production of volatiles from carotenoid precursors can be achieved through thermal (Crouzet and Kanasawud, 1992; Crouzet *et al.*, 2002) and various oxidative (Isoe *et al.*, 1969; Henry *et al.*, 1998) processes. In addition Bosser *et al.*, (1995) proposed a free radical pathway to ß-ionone from ß-carotene in a xanthine oxidase system. In an earlier related study, Bosser and Belin (1994) had demonstrated the production of free radicals in that system. The xanthine

oxidase system has additionally been used to obtain C-13 norisoprenod molecules as cleavage products of neoxanthin (Waché and Belin, 2000, Waché *et al.*, 2002). The authors claimed that cleavage in the 9,10 position was favoured.

In addition, micro-organisms have been demonstrated to have the potential to play a role in carotenoid degradation that might lead to post harvest generation of norisoprenoids, thus increasing the aroma and flavour contribution of these compounds to plant extracts. Sánchez-Contreras *et al.* (2000) demonstrated that micro-organisms isolated from marigold waste were able to degrade lutein to 7,8-dihydro-β-ionol, β-ionone, 7,8-dihydro-β-ionone, and 3-hydroxy-β-ionone. More recently a peroxidase occurring in the extracellular liquid of the edible fungus *Lepistra irinia*, yielded an enzyme capable of degrading carotenoids to β-ionone and C-10 carotenoid degradation products (Zorn *et al.*, 2003). The authors raised the possibility that this enzyme is a "potential tool" for both "flavour production" and "laundry applications".

Ionones and pigment compounds were demonstrated to be produced by the symbiotic relationship between plants and arbuscular mycorrhiza (Fester *et al.*, 2002; Strack *et al.*, 2003). Consideration of the potential to utilise this relationship in the production of C-13 norisoprenoids may be interesting. More directly relevant to this discussion, Rodríguez-Bustamante *et al.* (2005), in an article which demonstrated that a microbial mixture was able to produce C-13 norisoprenoid aroma compounds from lutein, discussed the importance of biotechnological processes and possible competitive advantages over similar extractives obtained from plants.

In relation to boronia it is important to consider the utilisation of any un-metabolised carotenoids as precursors for the generation of volatiles. As discussed earlier it is clear that post harvest increases in β-ionone occur (MacTavish and Menary, 2000) although the mechanism has not been clarified. However consideration of the potential for the development of biotechnological opportunity would depend on the accumulated levels of carotenoids both from the levels remaining in the flowers after harvest and any accumulation from post harvest processes. MacTavish (1995) made some qualitative assessments of carotenoids in boronia observing the presence of α-

carotene, β-carotene, neoxanthin and violaxanthin. However to date no quantitative assessment of carotenoid levels in boronia flowers has been made.

Quantification of the levels of C-40 carotenoid precursors, including β-carotene may help clarify both the potential use of these compounds and issues relating to the mechanism of C-13 norisprenoid biosynthesis. Whilst β-ionone is likely to be derived from β-carotene, the hydroxy norisoprenoids in boronia are structurally related to the xanthophylls and are also able to form glycosidic conjugates. The potential for the release of flavour compounds from conjugates precursors in boronia volatiles through biotechnological interventions will be considered in the following section of this review.

1.3 Conjugated Flavour and Aroma Compounds

1.3.1 Overview

Glycosides of flavour and aroma compounds are ubiquitous in the plant kingdom. Numerous general reviews (Stahl-Biskup *et al.*, 1993, Winterhalter and Skouroumounis, 1997; Sarry and Günata, 2004) and reviews covering a range of specific subjects including C-13 norisoprenoid glycosides in plant tissues (Winterhalter and Schreier, 1994); monoterpene glycosides (Stahl-Biskup, 1987; Mateo and Jiménez, 2001), flavour release from glycosides in wine (Williams *et al.*, 1992), biological activity (Dembitsky, 2004), and monoterpene release in grape and fruit juices (Maicas and Mateo, 2005) have been published. The main classes of secondary metabolites that form glycosidic volatiles are monoterpene alcohols, C-13 norisoprenoids, shikimates (phenolic compounds) with some representation from aliphatic alcohols (Sarry and Günata, 2004). The aglycones are usually linked to glucose as a β-D-glucopyranose and occur as monosaccharides, disaccharides or very occasionally trisaccharides (Sarry and Günata, 2004; Schreier, 1997). The different types of disaccharide glycosides are summarised in **table 1.1** and show the range of possibility with regard to the sugar moieties. Whilst Sarry and Günata (2004) claim that the primary glycosidic linkage is always to glucose, Schreier (1997) referred to the possibility of glycons that include pentoses and other hexoses. Additionally, Crouzet and Chassagne (1999) referred to studies that demonstrated the presence of galacto- and allo- pyranosides in thyme and liverwort respectively. Benzyl and acylated glycosides have also been reported (Crouzet and Chassagne, 1999) with malonyl glycosides being the most common acyl form.

Glycosylated volatiles have been demonstrated to perform a wide range of biological functions in plants (Sarry and Günata, 2004). Their role as either accumulation and storage forms of volatile compounds or transport forms of lipophilic compounds was reviewed by Winterhalter and Skouroumounis (1997). The authors discussed the possibility that glycosylation of volatiles may be important to protect the plant from toxicity due to the free volatiles and that storage within vacuoles may be facilitated. Epidermal oil glands on boronia petals have been described (MacTavish, 1995) and

the question of the need for glycosylation to facilitate storage is interesting. Certainly many lipophilic volatiles exist in boronia that do not have the structural capacity for glycosylation, including α-pinene, β-pinene and β-ionone (Weyerstahl *et al.*, 1995) and it would not be unreasonable to assume that these compounds are also stored in subcellular compartments which ensures effective separation from the metabolic pool.

Systematic name	**Trivial name**
6-O-α-L-arabinopyranosyl-β-D-glucopyranosides	Vicianosides[1]
6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides	Rutinosides[1]
6-O-β-D-glucopyranosyl-β-D-glucopyranosides	Gentiobiosides[1]
6-O-β-D-xylopyranosyl-β-D-glucopyranosides	Primeverosides[1]
6-O-β-D-apiofuranosyl-β-D-glucopyranosides	Acuminosides[2]
6-O-α-L-arabinofuranosyl-β-D-glucopyranosides	

Table 1.1 Types of diglycosides found in plants.
1. Trivial name referred to in Sarry and Günata, 2004. 2. Trivial name referred to in Wang *et al.*, 2001.

The role of glycosylation in facilitating transportation of otherwise non-polar aglycones to different parts of the plant was classically demonstrated by the work of Croteau (1987) with regard to the translocation of menthone. That compound is metabolised to neomenthol and glycosylated in peppermint leaves prior to transport to the rhizome where the compound undergoes further metabolism. In pursuit of an explanation for the role of glycosides, researchers have proposed that glycosides are synthesised in the leaves and transported to the flowers where they undergo hydrolysis by glycosidases (Winterhalter and Skouroumounis, 1997). The site of biosynthesis in boronia has not been explored previously and experimental data with regard to the differences in glycoside content in leaves and flowers may provide some important insights and allow further speculation on the role of these compounds in the plant.

Certainly, an understanding of the site of biosynthesis and accumulation may have application with regard to increasing the yield of flavour and aroma volatiles from boronia as a range of techniques exist for the release of these valuable compounds from glycosidic precursors. The main *in vitro* methods reported in the literature for the release of flavour and aroma compounds from glycosylated conjugates are acid hydrolysis and enzymatic transformations. The later category includes the use of commercial pectinase preparations isolated from fungal sources, plant glycosidases, and fungal isolates. Several reviews (Winterhalter and Skouroumounis, 1997; Crouzet and Chassagne, 1999; Sarry and Günata, 2004) summarised the types of microbial and plant glycosidases that have been studied with regard to the hydrolysis of glycosidically bound flavour precursors. A more detailed exploration regarding methods for the release of volatiles will be explored in a later section of this review.

1.3.2 The Aglycone Moiety

A review by Winterhalter and Schreier (1994) comprehensively described the glycoconjugates of C-13 norisoprenoids that had been positively identified from plant sources up to the time of review. They include glycosides of mono-oxygenated (ionols), di-oxygenated (ionols, ionones, megastigmanes and damescenones), and more complex higher-oxygenated C-13 norisoprenoids. The reviewed studies have found C-13 norisoprenoid glyco-conjugates in a variety of plants including in leaves, fruits and flowers. Most recent studies involving isolation and positive identification of C-13 norisoprenoid glycosides have been from plant leaves and details of these glycosidic conjugates are summarised in **table 1.2**.

These studies demonstrated the wide variety of C-13 norisoprenoids bound as glycosides and their presence is likely to be dependant on the structures of carotenoid precursors in the various plants. The important role of C-13 norisoprenoids as flavour and aroma compounds (Winterhalter and Schreier, 1994; Williams *et al.*, 1992) underlines the importance of glycosides of these compounds as precursor compounds. The C-13 norisprenoids found in boronia, and listed by Weyerstahl *et al.* (1995), that could be bound as glycosides include ionols (β-ionol,

Common name	Scientific name	C-13 norisoprenoids	Type of conjugate	Reference
Lulo leaves	*Solanum quitoense* L.	Ionol Megastigmanes Vomifoliol	glucosides	Osorio *et al.* 1999
Italian tree – leaves	*Cydonia vulgaris* Pers.	Ionol Vomifoliol	glucosides	De Tommasi *et al.* 1996
Shiraz leaves	*Vitis vinifera*	Megastigmane diols	glucosides	Skouroumounis *et al.* 2000
Sage leaves	*Salvia officinalis*	Ionols	β-D-glucopyranosides	Wang *et al.* 1998
Rose petals	*Rosa damascena*	Ionols	β-D-glucopyranosides	Straubinger *et al.* 1997
Tea leaves	*Camellia sinensis*	Ionol	disaccharide	Ma *et al.* 2001
Quince leaves	*Cydonia Oblongo* Mill.	Ionols	β-D-glucopyranosides	Lutz-Röder *et al.* 2002
Apple leaves	*Malus domestica*	3,4 Dihydroxy-7,8-dihydro-β-ionone and 3-oxo-α-ionol	β-D-glucopyranosides	Stingl *et al.* 2002
Botanbofu leaves	*Peucedanum japanicum* Thunb.	Hydroxy ionone	β-D-glucopyranoside	Hisamoto *et al.* 2004
Aerial parts of *P. hydropiper*	*Polygonum hydropiper* L.	3-Oxo-6-hydroxy-α-ionol (roseoside)	β-D-glucopyranoside	Murai *et al.* 2001

Table 1.2 Recent studies on the occurrence of C-13 norisoprenoid glycosides in plants.

7,8-dihydro-β-ionol, and 4-oxo-β-ionol) and ionones (4-hydroxy-β-ionone, 3-hydroxy-β-ionone, 3-hydroxy-5,6-dihydro-β-ionone, and 3-oxo-5,6-dihydro-βionol). The structures of these compounds are detailed in a later section of this review. A study by MacTavish *et al.* (2002) demonstrated increased levels of several of these compounds following incubation with endogenous enzyme fractions. However the levels of these compounds were low (personal communication, N. Davies) and more comprehensive studies should be undertaken. An assessment of any potential for commercial application will be important.

With regard to the aglycone moieties of conjugates the most widely studied group of compounds are the monoterpene glycosides. These compounds are important because many enzymatically released monoterpenols have sweet and/or floral aroma and flavour characteristics (Latrasse, 1991; Flament, 1991; Weyerstahl *et al.*, 1995). A seminal study by Francis and Allcock (1969) isolated the β-D-glucosides of geraniol, nerol and citronellol from rose flowers and measured the ratio of glycosides to free monoterpenols. Whilst not totally comprehensive, a review by Stahl-Biskup (1987) summarised much of the progress in this field over most of the next two decades and reported on "22 different monoterpene glycosides in 25 investigated plants". Since that review, the contribution of research information to this field has been prolific. The presence of monoterpene glycosides has been demonstrated, through either enzymatic release of the aglycones and / or direct identification of the glycosides, in a wide range of plants including from fresh herbs (Van den Dries *et al.*, 1989), tea (Guo *et al.*, 1993), hyssop (Schulz and Stahl-Biskup, 1991), and *Prunus* fruits (Krammer *et al.*, 1991).

The monoterpenols that have been identified in boronia are linalool, 8-hydroxy linalool, hotrienol, 7-hydroxy hotrienol, lilac alcohol, nerol, geraniol and 5-hydroxy linalool. There are additionally a range of cucurbates (methyl cucurbate, methyl epicucurbate and methyl diepicucurbate), aliphatic alcohols (eg. octanol, dodecanol and decanol) and simple phenolic compounds (eg. 2-methoxyphenol and 2-phenyl ethanol) that could possibly be present as glycosidic conjugates in boronia flowers. All of these compounds and their potential aroma and flavour contribution to the extract should be carefully considered.

1.3.3 Extraction and Identification of Glycosides

The most common method reported in the literature for obtaining a primary extract of glycosidic conjugates is methanolic extraction of the plant material followed by adsorption chromatography on Amberlite XAD-2 resin. This is based on the work of Günata *et al.* (1985) with grape aroma compounds. Variations of this method have been widely used for the extraction of extracts from plant materials including raspberry fruits (Pabst *et al.*, 1992), sloe tree leaves (Humpf and Schreier, 1992), lulo leaves (Osorio *et al.*, 1999), shiraz leaves (Skouroumounis *et al.*, 2000), tea leaves (Ma *et al.*, 2001) and apple leaves (Stingl *et al.*, 2002). In several instances this was associated with further treatment with PVPP to remove polyphenolic compounds (Pabst *et al.*, 1992; Skouroumounis *et al.*, 2000; Ma *et al.*, 2001). Other variations included treatment with basic lead acetate at neutral pH to deposit catechins and pigments from extracts of tea (Nishikitani *et al.*, 1996). An alternative to this method used by some researchers was the extraction of plant materials with various non-polar and polar solvents followed by partitioning between butanol and water (De Tommasi *et al.*, 1996; Wang *et al.*, 1998). The butanol fraction contained the glycosides.

Further fractionation of the primary extract has been achieved with column chromatography on common resins including Sepahdex LH 20, reversed phase C-18, and silica gel (Pabst *et al.*, 1992; Wang *et al.*, 1998; Nishikitani *et al.*, 1996); various forms of counter current chromatography including Multi Layer Coil (MLCCC - Osorio *et al.*, 1999; Straubinger *et al.*, 1997), rotation locular (RLCC – Humpf and Schreier, 1992) and Droplet (DCCC – De Tommasi *et al.*, 1996) methods. Depending on the earlier treatments final purification has been achieved using semi-prep or preparative HPLC on RP C-18 (Pabst *et al.*, 1992); acetylation of glycosides followed by flash chromatography and preparative HPLC before deacetylation (Humpf and Schreier, 1992; Osorio *et al.*, 1999; Straubinger *et al.*, 1997); and derivitisation of the glycosidic fraction followed by various forms of High Resolution Gas Chromatography (HRGC) (Bicalho *et al.*, 2000).

The initial task in these studies with regard to boronia glycosides will be to obtain a glycosidic extract for further testing and hydrolysis experiments. This laboratory is

well equipped for solvent extraction and column chromatography work and the possibility of obtaining an XAD-2 extract will be explored. There is however no high speed countercurrent chromatography equipment, which has been used extensively in other laboratories working with glycosidic conjugates of flavour and aroma compounds. The University of Tasmania does have excellent analytical capacity including HPLC / MS and GC /MS equipment which have potential for monitoring the suite of compounds related to this work.

1.3.4 Release of Glycoconjugates

The role of enzymes in flavour release has been investigated over several decades. Hewitt *et al.* (1956) were able to release flavour from 'precursors' in watercress using an enzyme preparation they termed 'flavorese'. This investigation was part of ongoing research into flavour release from naturally occurring 'precursors' for the processed food industry. Release of the aglycon moiety of glycosides from plants has been investigated extensively and these compounds can be released through acid hydrolysis and enzymatic cleavage with each method providing advantages and disadvantages depending on the glycoside content of the plant extract (Winterhalter and Skouroumounis, 1997). Given that (1) a range of monoterpenols and C-13 norisoprenoids have been found in boronia (Weyerstahl *et al.*, 1995), and that (2) many of these compounds have low odour thresholds and are considered to have unique flavour and aroma characteristics that are valuable to the food industry, it will be important to consider in detail the potential mechanisms for release of these conjugates from boronia.

Acid Hydrolysis

Numerous literature reports have detailed the release of flavour compounds using acid hydrolysis. Unlike the enzymatic release of flavour and aroma volatiles, acid hydrolysis can result in both molecular rearrangements and varying patterns of volatile release with different pH treatments (Williams *et al.*, 1982; Mateo and Jiménez, 2000). A review by Winterhalter and Schreier (1994) detailed the changes to several ionols and hydroxy ionones including the formation of 1,1,6-trimethyl-1,2-dihydro-naphthalene (TDN), which produces an off flavour reminiscent of kerosene,

from glucosides of 3-hydroxy-β-ionone, which has been detected in boronia (Weyerstahl *et al.*, 1995). Stingl *et al.* (2002) have reported that acid hydrolysis of the β-D-glucopyranoside of 3,4-dihydroxy-7,8-dihydro-β-ionone also leads to the formation of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). The authors solved this problem by incubating the glycosidic extract with baker's yeast, reducing the ionone to an ionol, and hence allowing formation of the more favourable vitispirane.

Biotransformation is also important in the release of flavour compounds from glycosidically bound monoterpene alcohols. Hotrienol (2,6-dimethyl-1,3,7-octatrien-6-ol), which has been described as having a very sweet and flowery note similar to lime tree blossoms (Flament, I.,1991), was the degradation product (along with the associated neroloxide) of simultaneous distillation-extraction at pH 3.0 of the glucopyranoside of 2,6-dimethyl-3,7-octadecane-2,6-diol (Wintoch *et al.*, 1993). It might be assumed that this is a favourable process, in the generation of flavour and aroma from plant extracts containing the precursor, as the diol is described by these authors as odourless. More generally in wines, acid hydrolysis has been discussed as a favourable process due to its simulation of the processes which occur in wine ageing (Mateo and Jiménez, 2000).

An examination of the monoterpene and C-13 norisoprenoid alcohols that could possibly form glycosidic conjugates in boronia allows some discussion. Three dioxygenated monotepenes have been detected in boronia. They are 8-hydroxy linalool (3,7-dimethylocta-1,6-diene-3,8-diol), 7-hydroxy hotrienol (3,7-dimethylocta-1,5-diene-3,7-diol), and 5-hydroxy linalool (3,7-dimethylocta-1,6-diene-3,5-diol). If these compounds were present as glycosidic conjugates in boronia there may be a capacity for the generation of more favourable mono-oxygenated terpenes as has been observed in other studies (Williams *et al.*, 1982; Engel and Tressl, 1983). This however would have to be balanced with any adverse effects that might result from unfavourable rearrangements of C-13 norisprenoid alcohols in boronia.

Endogenous Glycosidases

Endogenous glycosides have been demonstrated to have a role in the release of flavour and aroma compounds during ripening and commercial manufacturing processes of numerous plants and plant products. The action of these endogenous glycosides has been studied in detail with regard to the tea manufacturing process where flavour is released from the leaves during the withering process (Wang *et al.*, 2001). An endogenous glycoside has also been demonstrated to have a role in the release of vanillin during both the natural ripening and curing of vanilla beans (Odoux *et al.*, 2003). Additionally, higher levels of C-13 norisoprenoid aglycones were released when an extract from lulo leaves was incubated with an endogenous enzyme compared to the use of emulsin (β-D-glucosidase) (Osorio *et al.*, 2003).

Watanabe *et al.* (1993) found that enzyme extracts from the flowers of *Jasminium polyanthum* F., *Jasminium sambac* Ait., and *Gardenia jasminoides* E. were able to release volatiles from bound conjugates. Further, the enzyme was most active at the flower opening stage. The role of plant glycosidases in the release of flavour and aroma compounds at the time of flower opening was reviewed by Winterhalter and Skouroumounis (1997), and Watanabe (1997) proposed that glycosidase enzymes are induced at the time of flower opening. The occurrence of these enzymes may in some cases explain why free volatiles relative to the bound form have been found to increase during the later stages of flower and fruit development as demonstrated in roses (Francis and Allcock, 1969).

Whilst endogenous glycosidases have been proposed by MacTavish *et al.* (2002) it is unlikely that these enzymes will have an immediate use in commercial processes, relating to the release of glycosidic conjugates from boronia extracts, due to the need for extraction from the same flower material that requires the application. Furthermore Maicas and Mateo (2005), in a discussion of plant glycosidases that focussed on grapes, mentioned that β-glucosidases of vegetal origin have low activity towards monoglucosides of terpenes that are glycosylated through tertiary alcohol groups. Glucosides of linalool are an example and the aglycon is a known boronia compound (Weyerstahl *et al.*, 1995).

Microbiological Glycosidases

Micro-organisms play an important role in natural processes. Human ingenuity, since Neolithic times, has taken advantage of these relationships to utilise bacteria and fungi in the production of foods which enhance flavour and aroma characteristics (Scharpf *et al.*, 1986). The action of yeast in bread-making leads to the production of volatiles which contribute to the "yeasty note of the crumb and cracker-like note of the crust" (Grosch and Schieberle, 1991). Volatile composition is affected in beer by the choice of yeast strain (Angelino, 1991) and in cheeses by both the starter culture and secondary microbial flora (Scharpf *et al.*, 1986).

In the wine industry release of glycosidically bound flavour and aroma compounds by micro-organisms has been thoroughly investigated with much of the available information reviewed by Maicos and Mateo (2005). Mateo and Jiménez (2000) reviewed the hydrolytic release of monoterpenes from wine glycosides through acid and enzymatic hydrolysis. The enzymatic release was achieved with endogenous grape glycosidases, yeast glycosidases and exogenous fungal preparations (Mateo and Jiménez., 2000). D'Incecco *et al.* (2004) found that the commercial wine bacteria, *Oenococcus oeni* strain Lalvin EQ54, exhibited disaccharidase activity during malolactic fermentation. Endogenous fungi have been tested for their ability to break down glycosides in the presence of ethanol and glucose (Belancic *et al.*, 2003). The authors isolated an extracellular β-glucosidase from a *Debaryomyces vanrijiae* isolate that was able to metabolise the release of monoterpenols from grape glycosides.

The use of microbial isolates with glycosidase activity in the wine industry during fermentation must take into account the low pH and alcohol content of the fermenting juice. Equivalent problems are unlikely to exist with regard to the use of micro-organisms for the release of conjugates in boronia and it has been reported that boronia flowers show obvious signs of microbial growth both after harvest and during harvest in some weather conditions (Personal communication, R. Menary) The possibility exists of an endogenous micro-organism that may be able to metabolise glycosides in boronia *in vivo*.

A more common approach with regard to the hydrolysis of plant glycosides with microbial enzymes is through the use of pectinase preparations prepared from fungal sources. Fungal pectinase preparations, with wide ranging glycosidase activity, have been used to release flavour and aroma volatiles from many plant products and several commercially available products are available. **Table 1.3**, reproduced from Sarry and Günata (2004), lists some of the pectinases that are commonly reported in the literature to be effective in the release of bound volatiles.

Pectinase	**β-glucosidase activity**	**α-arabinofuran-osidase activity**	**α-rhamnosidase activity**	**β-apiosidase activity**
Cellulase A	6.1	0.6	0.007	nd
Hemicellulase	7.1	7.0	0.9	nd
Pectinol VR	0.2	0.1	nd	nd
Rohament CW	3.3	0.7	0.4	nd
Pectinol D5S	0.5	0.7	nd	nd
Pektolase 3PA	1.5	3.8	0.04	0.3
Ultrazym 100	0.5	0.1	nd	0.03
Pectinase 263	7.2	1.4	0.3	0.2
AR 2000	5.6	9.2	0.32	1.08

Table 1.3 Glycosidase activities (nkat/mg) of various commercial enzyme preparations. Reproduced from Sarry and Günata (2004).
Source of enzymes: Gist Brocades – Cellulase A, Hemicellulase, Pectinase 263, AR 2000. Röhm – Pectinol V, Rohament CW, Pectinol D5S. Grinsted – Pektolase 3PA Ciba-Geigy – Ultrazym 100. nd = not detected

The different commercial pectinases available have widely varying specific glycosidase activities and several studies have looked closely at these variable activities. Winkel and Boesveld (2000) compared three different commercial enzyme preparations (Biopectinase OKL, Biopectinase CT and Aromazyme) with regard to the release of volatiles from tea. The authors found significant differences in

different glycosidase activities and perhaps more importantly were able to demonstrate that this translated to different flavour and bitterness outcomes. It was suggested that the Biopectinase CT was slightly more selective with regard to release of flavour preferable volatiles.

Commercial pectinases are used routinely in the wine industry where bound volatiles are often found at higher concentrations than the free compounds (Williams *et al.*, 1992). The release of volatiles from various plant sources using commonly available enzyme preparations is presented in **table 1.4**. The results of these studies demonstrated the release of aglycones including monoterpenols, C-13 norisoprenoids, shikimates, aliphatic alcohols and furanones from a wide range of plant products. However the generation of free volatiles using fungal preparations is not straightforward. Sefton and Williams (1991) demonstrated that the hydrolysis of glycosidic conjugates using fungal enzyme systems led to the production of oxidation artefacts. In addition, monoterpene polyols, often released through enzymatic hydrolysis, are regarded as flavour inactive (Strauss *et al.*, 1986). These compounds would require an additional treatment or alternative methodology for release to be converted to more valuable flavour compounds. The flavour activities of monoterpene polyols and monoterpenols will be dealt with in more detail in a later section.

Overall the literature indicated that, whilst demonstrating release of volatiles, outcomes from the use of commercial pectinase preparations need to take into account the complexities of variable specific glycosidase activity (Sarry and Günata, 2004; Winkel and Boesveld, 2000), the presence of other enzymes such as esterases (Roscher *et al.*, 1997), tannases (Winkel and Boesveld, 2000) and an apparent ability to cause oxidation of released volatiles (Sefton and Williams, 1991). It would seem appropriate to use a broad spectrum pectinase in experiments with boronia.

Commercial pectinase	Plant	Scientific name	Compound class released	Reference
Hemicellulase and emulsin	Passion Fruit	*Passiflora* species	A, B, C, D	Chassagne *et al.* 1999
Hemicellulase and emulsin	Tropical Fruit - Cupuaçu - Bacuri - Acerola		 A, C, D A, B[1], C, D A, B, C, D	Boulanger et al. 2000
Hemicellulase and emulsin	Mango fruit (Kensington Pride)	*Mangifera indica* L.	A, B, C, D	Lalel *et al.* 2003
Pektolase 3PA	Muscat of Frontignan	*Vitis vinifera* L.	A, B, C, D	Bureau *et al.* 2000a
Pektolase 3PA and hemicellulase	Wine Grapes Muscat of Alexandria Shiraz	*Vitis Vinifera* berries leaves berries leaves	 A, B, C, D A, B, C, D A, B, C, D A, B, C, D	Wirth *et al.* 2001
Rohapect D5L	Cape Gooseberry	*Physalis peruviana* L.	C, D	Mayorga *et al.* 2001
Rohapect D5L	Lulo fruit pulp and peelings	*Solanum vestissimum* D. pulp peelings	 A, B, C, D A, B, C, D	Suárez *et al.* 1991
Rohapect D5L	Melón de Olor fruit	*Sicana odorifera*	C, D	Parada *et al.* 2000
AR 2000	Muscadet juice	*Vitis Vinifera* L. cv. Melon B.	A, B, C[2], D[2]	Schneider *et al.* 2001
AR 2000	Muscadine grape juice	*Vitis rotundifolia* Michx cv. Carlos	A, C, D, E[3]	Baek and Cadwallader, 1999
AR 2000	nectarines	*Prunus persica* L. Batsch var. *nucipersica* cv. Springbright	A, B, C, D	Aubert *et al.* 2003

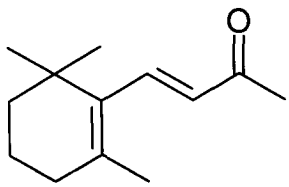
Table 1.4 Results of hydrolysis of plant glycosidic extracts with commercial pectinase preparations. The classes of flavour and aroma compounds reported as being released in these studies are; A = monoterpenes, B = C-13 norisoprenoids, C = Shikimates (aromatic alcohols), D = Aliphatic alcohols, E = Other significant classes. (1. Minimal amounts compared to other compound classes. 2. The aliphatic and aromatic alcohols were not quantified in this study but are indicated as present through annotation of a GC-MS trace. 3. Furanones).

1.4 The Chemical Composition of Boronia Extract

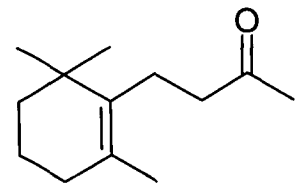
Understanding the chemical make-up of boronia is critical to the process of developing new technology that might increase the yield and quality of the extract. Research in this field dates back to 1927 with the identification of β-ionone in the extract by Penfold (Cited by Davies and Menary, 1983). The first comprehensive study was undertaken within the Tasmanian Horticultural Research Group during the late 70's and early 80's (Davies and Menary, 1983). In that study the authors identified 77 compounds including monoterpenes, monoterpene alcohols, several C-13 norisoprenoids, jasmonates and numerous longer chain hydrocarbons (C-17 to C-33) out of over 150 compounds detected.

Studies during the early nineties by Weyerstahl's group led to the positive identification of 128 compounds including over twenty norisoprenoids (Weyerstahl *et al.*, 1994; Weyerstahl *et al.*, 1995). β-Ionone was found at high concentration in the extract with smaller amounts of 7,8-dihydro-β-ionone and the isomers of 3-hydroxy megastigma-7-en-9-one. Weyerstahl *et al.* (1995) identified a further seventeen C-13 norisoprenoids which were found at still lower concentrations. Kaiser (2002) has additionally reported (+)-theaspirane A, (+)-theaspirane B, and 5,8-epidioxy-6-megastigmen-9-ol. He suggested, that (i) the theaspiranes might be formed through photoisomerisation of α-ionone and subsequent reduction, and that (ii) the epidioxy molecule may be formed from β-ionol through a 1,4-addition of oxygen. The only other norisoprenoid that does not contain 13 carbons so far identified in boronia is 4,5-epoxy-γ-homocyclocitral which Weyerstahl *et al.*, (1995) suggested was an artefact.

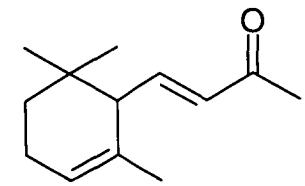
It is important to consider the structure of the norisoprenoid molecules in boronia with regard to their homology with possible parent C-40 carotenoids. **Figure 1.4** details the chemical structures of the C–13 norisoprenoids found in boronia as described by Weyerstahl *et al.* (1995) with additional detail from Kaiser (2002). Compounds 8, 9, 12, 14, 17, 18 and 19 have the capacity to form glycosidic conjugates.



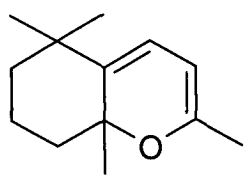
1. β-ionone



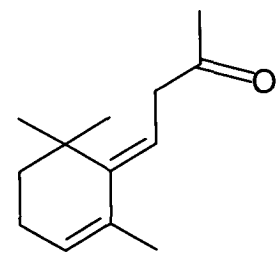
4. 7,8-dihydro-β-ionone



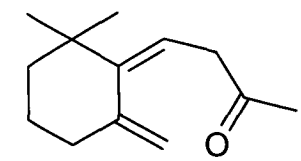
3. α-ionone
(E and Z isomers)



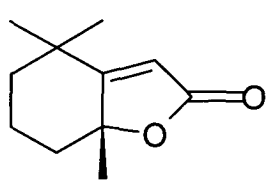
4. cyclic β-ionone



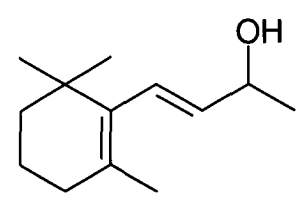
5. (Z)-retro-α-ionone



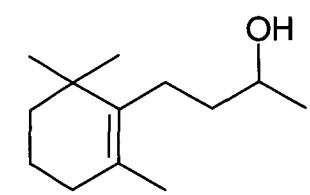
6. (Z)-retro-γ-ionone



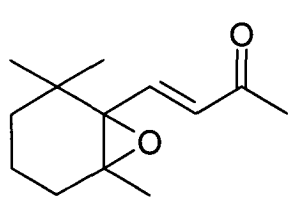
7. dihydroactinidiolide



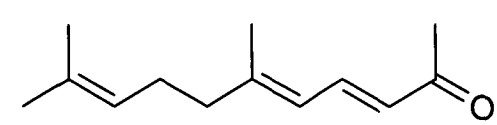
8. β-ionol



9. 7,8-dihydro-β-
ionol



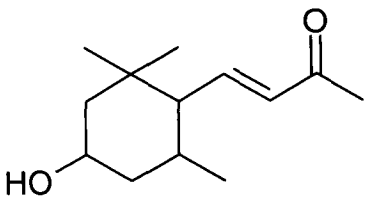
10. 5,6-epoxy-β-ionone



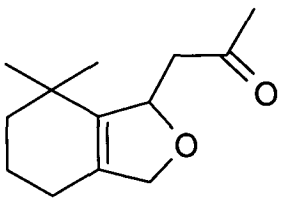
11. (E, E)-pseudo-ionone

Figure 1.4 The structures of norisoprenoid volatiles that have been identified in extracts from boronia.

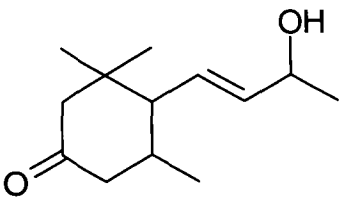
Figure 1.4 (cont.) Norisoprenoids found in boronia



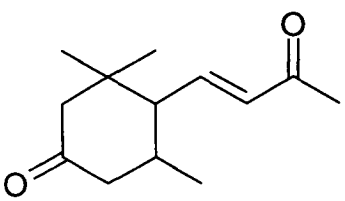
12. 3-hydroxy-5,6-dihydro-β-ionone



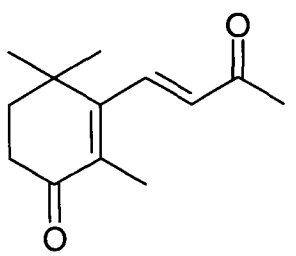
13. 7,11-epoxymegastigm-5(6)-en-9-one



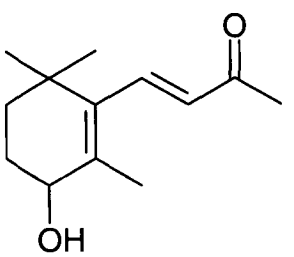
14. 3-oxo-5,6-dihydro-β-ionol



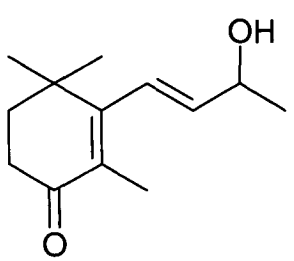
15. 3-oxo-5,6-dihydro-β-ionone



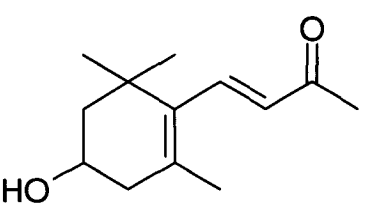
16. 4-oxo-β-ionone



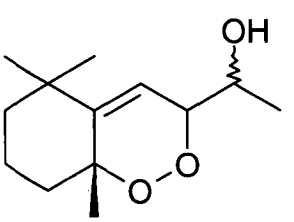
17. 4-hydroxy-β-ionone



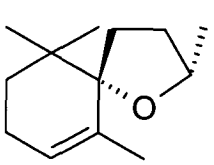
18. 4-oxo-β-ionol



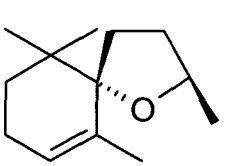
19. 3-hydroxy-β-ionone



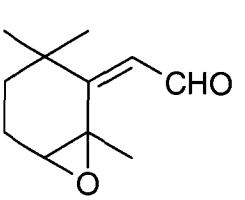
20. 5,8-epidioxymegastigm-6-en-9-ol



21. (+)-theaspirane A



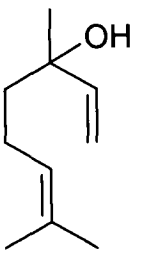
22. (+)-theaspirane B



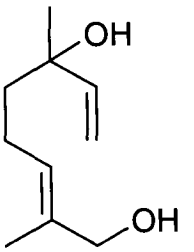
23. 4,5-epoxy-homocyclocitral

The structures of monoterpenols which also have the capacity to form conjugates are detailed in **figure 1.5**. Several of these compounds have been described as having floral aroma sensations (Weyerstahl et al., 1995; Latrasse, 1991) and have the potential to be an important class of aroma and flavour compounds in boronia. The comprehensive chemical analysis of compounds found in boronia has allowed the formation of in-house MS libraries, which will aid in the identification and screening of released aroma volatiles from conjugates.

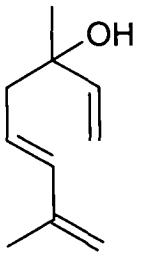
By way of explanation ionones are often named in the literature using a megastigmane numbering system. This is clarified along with the numbering system for these compounds in **Appendix 1** and the compound referred to by Weyerstahl as 3-hydroxymegatigm-7-en-9-one could alternatively be named as 5,6-dihydro-β-ionone.



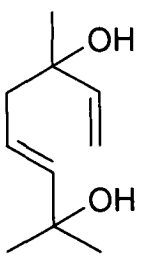
24. Linalool



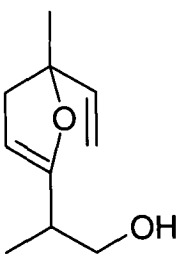
25. 8-Hydroxy linalool (3,7-dimethylocta-1,6-diene-3,8-diol)



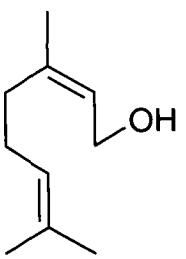
26. Hotrienol



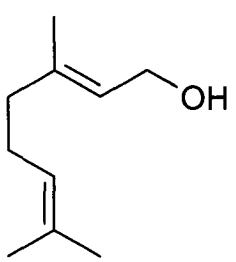
27. 7-Hydroxy hotrienol (3,7-dimethylocta-1,5-diene-3,7-diol)



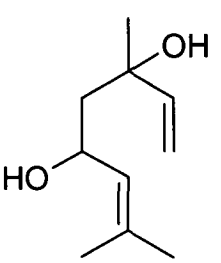
Lilac alcohol



Nerol



Geraniol



5-Hydroxy linalool

Figure 1.5 The structures of monoterpene alcohols identified in boronia

1.5 Aroma Characteristics of Boronia and its Components

Arctander (1960) described boronia absolute as "fresh fruity-green, sweet tea-like, slightly spicy-herbaceous (reminiscent of cinnamon and tobacco leaf)". He went on to say that, "the main body notes display a tremendously rich and tenacious floral undertone of warm, woody-sweet character". This layered complexity mirrors the range of compounds identified in boronia (Weyerstahl *et al.*, 1994; Weyerstahl *et al.*, 1995), which include, monoterpenols, sesquiterpenes, esters (including dodecyl acetate), C-13 norisoprenoids, methyl jasmonates, and long chain hydrocarbons with lower concentrations of hydrocarbon monoterpenes, aromatic and aliphatic alcohols, and cucurbates. There are additionally several groups of compounds that elute late in the GC profile (after octadecane) that are not normally taken into account when the extract is analysed. These compound groups include the tiglamides, 8-hydroxy linalyl esters, and various derivatives of cinnamic acid which make up over 35% of the absolute. The contribution of these "late eluting" compounds to the overall aroma and flavour characteristics is unknown.

There is limited information about the flavour and aroma characteristics of specific compounds in the literature. However, **table 1.5** details some boronia compounds and their specific aroma characteristics that might contribute to the overall aroma profile described by Arctander (1960). A consideration then of the compounds that might be increased through release from precursors, including C-40 carotenoids and glycosidic conjugates, provided some context for this investigation.

The C-13 norisoprenoids as a group are generally regarded as important flavour and aroma compounds with low flavour thresholds (Winterhalter and Schreier, 1994). However whilst numerous studies have reported on the low flavour and aroma threshold of β-ionone and its unique aroma characteristics in this regard (Guenther, 1949) much less has been reported for the other C-13 norisoprenoids found in boronia. The various ionones (C-13 norisoprenoids) might be expected to contribute floral, fruity and woody elements to boronia extract commensurate with the existing knowledge with regard to C-13 norisoprenoids. 7,8-Dihydro-β-ionone has been claimed to have a "fruity, ionone-orris-like smell" (Weyerstahl *et al.*, 1994).

Compound	Aroma characteristic	Kovats' Index	Threshold in water (mg/kg)	Reference
β-pinene	Citrus, terpene like	976		Rega *et al.* 2003
β-myrcene	Peel, unpleasant, geranium, medicine	985		Rega *et al.* 2003
limonene	Fruity, lemon, anise	1027		Rega *et al.* 2003
Benzyl Alcohol	Cloudberry-like	1008		Latrasse, 1991 (p. 361)
2-Phenylethanol	Slightly rose-like	1088	0.086	Latrasse, 1991 (p. 361)
Hotrienol (24)	sweet and flowery note similar to lime-tree blossoms	1088		Flament, 1991 (p. 625) Weyerstahl *et al.* 1995 (p. 307)
Linalool (24)	Floral	1088	0.006	Latrasse, 1991 (p. 361)
8-hydroxy linalool (26)	fatty, green herbaceous, woody, citronella-like	1341		Weyerstahl *et al.* 1995 (p. 307)
7,8-dihydro-β-ionone	fruity, ionone-orris-like	1424		Weyerstahl *et al.* 1994 (p. 1043)
7,11-epoxymegastigm-5(6)-en-9-one	(+) – sweet, food-like, slightly vanillic note. (-) – weak, somewhat floral-fruity, raspberry, ionone like	1556		Brenna *et al.* (2005)
β-ionone	Warm, woody, somewhat dry odour with a fruity undertone (raspberry)	1475	0.0013	Tan and Siebert, 2004 Arctander, 1960 (p.1778)
Dihydroactinidiolide	Fruity odor	1496		Latrasse, 1991 (p.358)
Theaspirane B	Fruity, floral			Wang *et al.* 2005
β-ionol	Fruity	1406		Morales and Duque, 2002
4-hydroxy β-ionone	Woody, green	1642		Morales and Duque, 2002

Table 1.5 Literature descriptions of some boronia compounds. The Kovats' indices are those published by Weyerstahl *et al.* (1995). Where reported the aroma thresholds are published in the first mentioned reference.

However, Wirth *et al.* (2003) without providing references, referred to several polyoxygenated C-13 norisoprenoids, including hydroxy compounds found in boronia, as being odourless or less odourous than β-ionone. In contrast, studies on the flavour and aroma characteristics of C-13 norisoprenoids in tobacco referred to a range of di-oxygenated C-13 norisoprenoids, including 3-hydroxy-β-ionone, as having "excellent aroma" (Fujimori, *et al.*, 1976).

Many studies using GC-Olfactometry techniques, which referred to the importance of aroma impact of norisoprenoids (Mahattanatawee *et al.*, 2005, Culleré *et al.*, 2004; Wang *et al.*, 2005) often only provide detail for the main norisoprenoids which include β-ionone, α-ionone, β-damascenone and β-cyclocitral which have well characterised aromas. Unfortunately there is much less information in the literature about the specific characteristics of the hydroxy C-13 norisoprenoids that may potentially be released from both carotenoid and glycosidic precursors. A recent study by Morales and Duque (2002) provided some insight. These authors presented olfactory information for 4-hydroxy β-ionone and β-ionol which are described as woody/green and fruity respectively. These odour characteristics would make beneficial contributions to the boronia extract.

Skouroumounis and Sefton (2000) referred to the hydroxylated β-damascenone, 3-hydroxy damascone, as flavourless; whilst Wirth *et al.* (2001) made the claim that this compound is "an important norisoprenoid aglycon of grape berries". It may be necessary to consider, as is the case for the monoterpenes, whether some polyoxygenated C-13 norisoprenoids have less aroma importance than the mono-oxygenated aldehydes such as β-ionone.

Monoterpene hydrocarbons, including α-pinene, β-pinene and limonene, are extract volatiles routinely measured as part of the quality control process for boronia production. These compounds are not regarded as imparting "a favourable effect on the aroma" (Plummer *et al.*, 1999) and are unlikely to be released from precursors. However monoterpene alcohols including linalool, which are currently present in low concentrations (Weyerstahl *et al.*, 1995) in the extract are more likely to impart

favourable effects on the extract due to their floral characteristics. They are an essential flavour component of tea (Flament, 1991), and Latrasse (1991) discussed the importance of monoterpene and sesquiterpene alcohols to the odour characteristics of berry fruits. In blackberries monoterpenols are responsible for the "intense flowery and fruity notes" (Latrasse, 1991). Release of monoterpenols then (see **figure 1.5** for structures), from glycosidic conjugates have the potential to contribute to the floral notes of the extract. The poly-oxygenated monoterpenol 8-hydroxy linalool, might accentuate the green herbaceous notes.

Other polyoxygenated monoterpenes such as the hydroxylated linalool metabolites found in many plants including papaya and passionfruit (Winterhalter, 1991) are regarded as flavour precursors with the ability to be transformed to aroma monoterpenes through both chemical and enzymatic means (Williams *et al.*, 1982; Winterhalter, 1991). In relation to this, (and as discussed in the glycoside section of this review), it is widely recognised that monoterpene polyols can be converted to flavour compounds through acid hydrolysis of the glycosidic conjugates.

In addition to glycosidic compounds, possible precursors in boronia for linalool related compounds include a series of 8-hydroxylinalyl esters (Weyerstahl *et al.*, 1995). The 8-hydroxy linalyl esters identified by that author are listed in **table 1.6**.

8-hydroxy linalyl acetate	8-hydroxylinalyl octonoate
8-hydroxylinalyl isobutyrate	8-hydroxylinalyl octonoate
8-hydroxylinalyl 2-methylbutyrate	8-hydroxylinalyl nonoate
8-hydroxylinalyl 3-methylbutyrate	8-hydroxylinalyl decanoate
8-hydroxylinalyl valerate	8-hydroxylinalyl undeconoate
8-hydroxylinalyl tiglate	8-hydroxylinalyl dodeconoate
8-hydroxylinalyl heptonoate	8-hydroxylinalyl trideconoate

Table 1.6 8-Hydroxy linalyl esters found in boronia extract as detailed by Weyerstahl *et al.* (1995)

1.6 Searched-for Outcomes and Experimental Design

The primary question posed in this thesis concerns the potential for boronia to be processed in order to provide an increased yield of volatile compounds that can contribute to the quality and overall yield of commercially available boronia extract. This will be achieved through:

1. Developing an understanding of the metabolic pathways, and occurrence of compounds, that yield so far unutilised volatile precursors. In particular the study will focus on the metabolism of C-40 carotenoids that are likely precursors of C-13 norisoprenoids and on glycosidic precursors of C-13 norisoprenoid alcohols, monoterpenols and other alcohol volatiles known to be present in the extract.

2. The investigation and development of potential post harvest processes that will yield volatiles from the carotenoid and glycosidic precursors studied in the first part of this study.

The investigation will be undertaken with consideration for the potential of the various compounds to contribute to the extract's yield and quality given the desired characteristics and necessary continuity of the aroma and flavour profile of the commercially available extract.

In addition, it is intended to increase the database of chemical, biochemical and processing knowledge developed over two decades of research by the University of Tasmania and strongly contributed to in the area of boronia chemistry by Weyerstahl *et al.* (1994) and Weyerstahl *et al.* (1995) at the Institut für Organische Chemie. This new knowledge, along with the existing database, has the potential to be the basis of future research in clonal selection, post harvest incubation, and processing technology.

Figure 1.6 gives an overview of the relationship between precursor secondary metabolites and the volatiles that can be potentially released from boronia flowers. C-40 carotenoids have been accepted as the metabolic precursors of C-13 norisoprenoids for several decades (Winterhalter and Rouseff, 2002). Cleavage of hydrocarbon carotenoids in the 9,10 position can lead to the production of several high value C-13 norisoprenoids. These include β-ionone which contributes strongly to the flavour and aroma of boronia extract. Cleavage of xanthophylls in the same

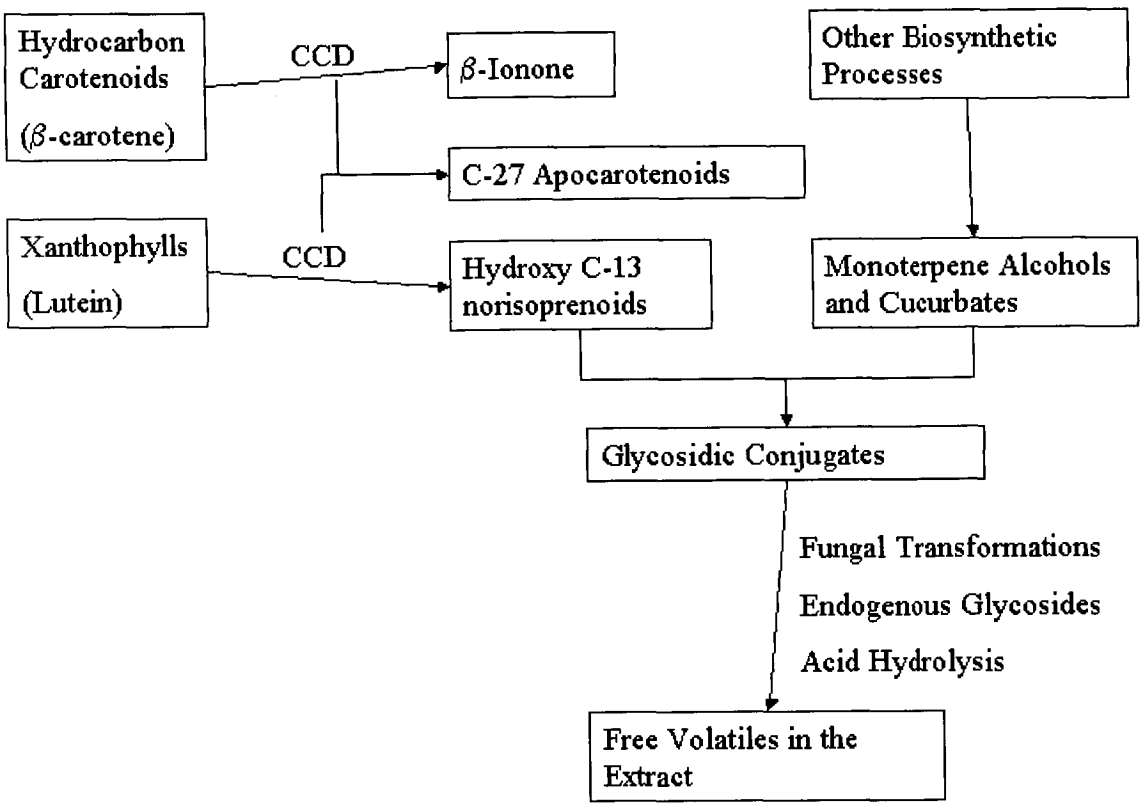


Figure 1.6 Schematic representation of study areas. (CCD = Carotenoid Cleavage Dioxygenase)

position can lead to the bio-generation of poly-oxygenated C-13 norisoprenoids. This group of molecules includes hydroxylated C-13 norisoprenoids which, along with monoterpenols, cucurbates (the alcohol analogues of methyl jasmonates) and other alcohols, are able to form flavourless glycosidic conjugates (Crouzet and Chassagne, 1999; Winterhalter and Skouroumounis, 1997).

These conjugated compounds are not available as aroma and flavour compounds due to (1) their reduced solubility in the non-polar solvents that are routinely used for extraction procedures and, (2) a lack of any sensory characteristics in the bound form. Aglycones can then be released from the conjugates by several different mechanisms to yield further valuable flavour and aroma volatiles not previously available to the extract.

The concurrence of several different factors has allowed this study to be undertaken. They are:

(1) an ongoing boronia industry and previous research conducted over two decades which includes the comprehensive knowledge of boronia volatiles as published in the two Weyerstahl papers.

(2) the availability of GC-MS and HPLC-MS with both diode array and MS (APCI) detection systems.

(3) the large body of information in the literature with regard to detection and release of bound volatiles.

(4) anecdotal evidence that boronia marc continues to emit volatiles after commercial extraction (personal communication; Menary, 1999).

A strong part of the rationale for this work relates to the balance between the flavour and fragrance market's need for natural flavours (Cheetham, 1997) and the development of low cost synthetic replacement products (Mookherjee *et al.*, 1995). Manipulation of precursors to yield important boronia volatiles has the potential to increase the yield and market differentiation of boronia. In particular, the high cost of boronia absolute and the availability of synthetic options prevent the use of this wonderfully complex extract from being used in the perfumery industry. Furthermore, research into the production of plant secondary metabolites using biotechnological processes includes the possibility of inserting genes into micro-organisms for conversion of readily available precursors to high value compounds (Verpoorte et al., 2002). With respect to high value secondary metabolites in boronia there is currently a German based technological project to insert a site specific carotenoid cleavage dioxygenase into micro-organisms for the production of C-13 norisoprenoids (Personal communication, Schwab, 2003). Such developments have the potential to have an impact on the economic value of boronia extract. Hence any increase in yield through further improvements in post harvest technologies would be invaluable.

Chapter 2
Identification of C-27 Apocarotenoids

2.1 Introduction

In the literature review evidence that C-40 carotenoids are precursors of ionone compounds was discussed with regard to structural considerations. Given the important contribution of β-ionone and other C-13 norisoprenoids to the flavour and aroma of extracts from boronia it is appropriate to begin this study by focussing on evidence for the biosynthesis of C-13 norisoprenoids from carotenoid precursors. Note that this chapter has been previously published (Cooper *et al.*, 2003).

2.2 Materials and Methods

2.2.1 Materials

Boronia megastigma (Nees) clone 3 flowers developed by the University of Tasmania and grown in southern Tasmania were used. All chemicals and solvents were analytical grade.

2.2.2 Carotenoid Extraction from Boronia Flowers

Typically 10-20 g of flower material was extracted using a method adapted from that of Jarén-Galán *et al.* (*1999*). Accordingly 20 g of boronia flowers or buds, plus 5.0 g of calcium carbonate for acid neutralisation (Mercadante, 1999b; Breithaupt and Bamedi, 2002), was homogenised in 100 mL of acetone (4°C) for 60 s using an Ultra-Turrex (T25 basic, Ika labortechnik, setting 6), fitted with an 18 mm head. The mixture was centrifuged for 5 min at 15,000 g (Beckman J2 – 21 M/E, rotor

20.0). The pellet was extracted twice more in 50 mL of acetone and the supernatants combined in a separating funnel with 100 mL of diethyl ether. The mixture was shaken and 200 mL of 10% NaCl was added to promote separation of the ether layer. The aqueous layer was discarded and the ether layer washed several times with 100 mL aliquots of 10% NaCl. The ether fraction containing the pigments was dried over sodium sulfate, filtered, and the solvent removed by rotary evaporation. The pigments were stored at –70°C prior to analysis. All operations were at 4°C under reduced or pale yellow light (Van Vliet *et al.*, 1996).

2.2.3 HPLC Analysis

A Waters Alliance 2690 HPLC and a 996 Photodiode array detector were used for chromatography and identification of carotenoids and apocarotenoids. A Waters Nova-Pak 150 x 3.9 mm i.d. C18 column fitted with an Alltech Econosphere C18 guard cartridge was used to achieve separation. Three different programs were used for analytical purposes.

Program A: Initial conditions were 87% acetonitrile/10% methanol/3% water for 3 min programmed to 85% methanol/15% hexane at 10 min which was then isocratic for 5 min. The flow rate was 1 mL/min. Following the completion of each run the column was returned to starting conditions over 1 min and equilibrated for 8 min prior to the next run.

Program B: Initial conditions were 50% acetonitrile/50% water for 2 min followed by a linear gradient to 85% acetonitrile/15% methanol at 15 min. A further linear gradient to 85% methanol/15% hexane at 25 min was then held for 10 min. The flow rate was 1 mL/min. The column was re-equilibrated to start conditions for 10 min between samples.

Program C: Starting conditions were 50% acetonitrile/50% water for 2 min. This was followed by a linear gradient to 85% acetonitrile/15% methanol at 22 min and a further programme to 85% methanol/15% hexane at 32 min which was held for 8

min. The flow rate was 1 mL/minute. Re-equilibration was to 100% methanol for 3 min and 50% acetonitrile/50% water for 9 min.

Data Processing

Waters Millenium software was used to analyze data and chromatograms were extracted at 430 and 451 nm. Data was recorded from 250–700 nm every 1 s at 1.2 nm resolution. Lutein was identified on the basis of published UV-Vis data and retention times.

2.2.4 HPLC/MS

Mass spectral data was obtained with a Finnigan LCQ equipped with an Atmospheric Pressure Chemical Ionization (APCI) ion source. Settings were sheath gas 60 psi, auxiliary gas 15 psi, vaporiser temperature 450°C, discharge current 6 μA, capillary temperature 170°C, capillary voltage 20 volts, default MS/MS collision energy 25%. Scanning usually occurred over the *m/z* range 100 to 1200. Signals for the apocarotenoids reported here were maximised by shortening the *m/z* range to 100-680.

2.2.5 Synthesis of Diazomethane

Diazomethane was prepared in a 250 mL conical flask by adding *N*-methyl-*N*-nitrosourea (3.0 g, 22.5 mmol) in small portions to a mixture of aqueous potassium hydroxide (40% w/v, 40 mL) and diethyl ether (50 mL) at 5°C with constant stirring (magnetic stirrer) for 20-30 min. The ether layer was separated and the aqueous layer further extracted with 25 mL of ether. The combined ether fractions were allowed to stand over some solid potassium hydroxide (30 min minimum) and then transferred to a screw top bottle for storage at −15°C.

2.2.6 Methylation of Carotenoid Extracts

Aliquots of carotenoid extract (10 mg) were treated with diazomethane (1 or 2 mL of the ether fraction above) for 30 min after which the solvent was evaporated under a stream of nitrogen. The resultant methylated extract was redissolved in 2 mL of acetone and analyzed by HPLC. This process was conducted under low or nil light conditions.

2.2.7 Safety

Caution: Diazomethane is a highly toxic, yellow carcinogenic gas that must be handled in a fume cupboard. The gas is explosive but may be used safely as a solution in diethyl ether. Nevertheless it should be treated with great caution. Due to its potential carcinogenic nature and high potency as a skin irritant, all necessary safety protocols for handling dangerous compounds should be strictly followed.

2.3 Results and Discussion

Five C-27 apocarotenoids were identified for the first time in the flowers of *Boronia megastigma* (Nees), using HPLC with diode array detection and HPLC-MS. This identification was on the basis of published UV-Vis spectroscopic data, HPLC-MS data, and changes following methylation.

Initial chromatography of acetone extracts of boronia flowers yielded five peaks with UV-Vis spectra that had single absorption maxima between 428 and 440 nm (**Figure 2.1**). These UV-Vis maxima were consistent with those previously reported for C-27 apocarotenoids. Data reported by Singh *et al.* (1973) included ß-apo-10'-carotenoic acid (λ_{max} = 425 nm in light petroleum) and ß-apo-10'-carotenal (λ_{max} = 437 nm in light petroleum). Yokoyama and White (1966) reported 3-hydroxy-ß-apo-10'-carotenal as having a similar spectrum to ß-apo-10'-carotenal, both consistent with

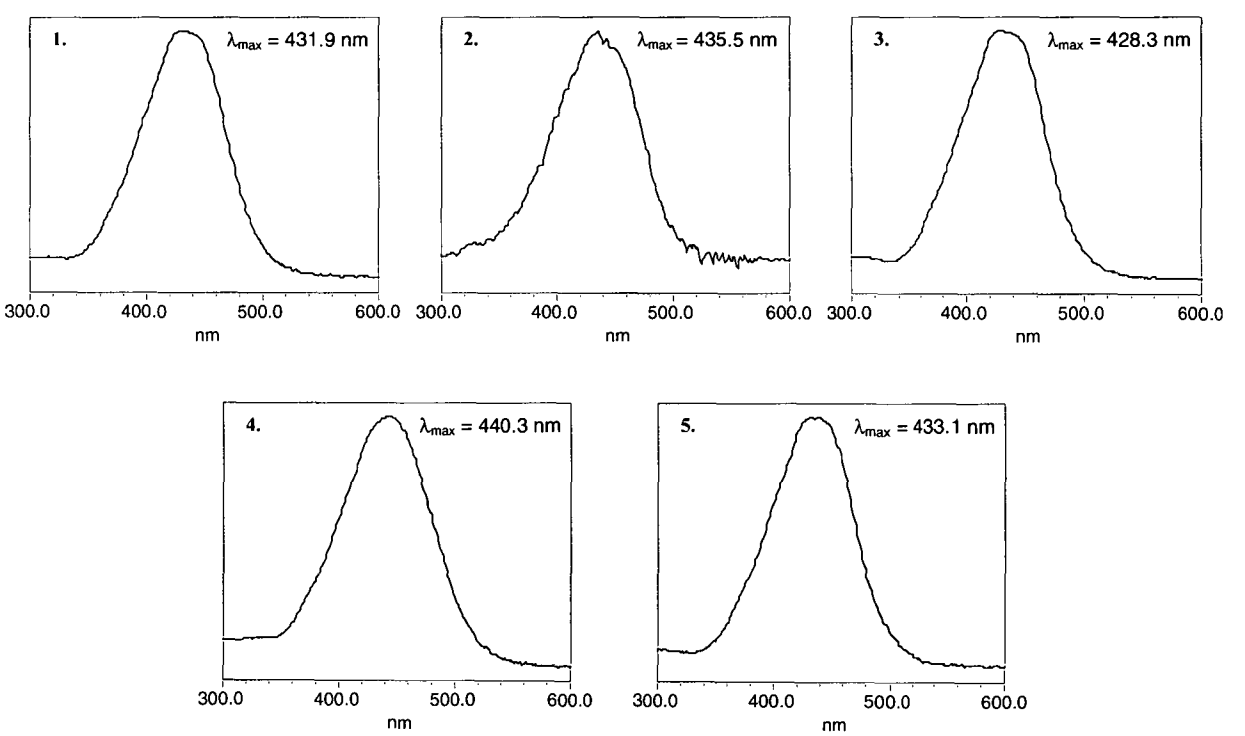
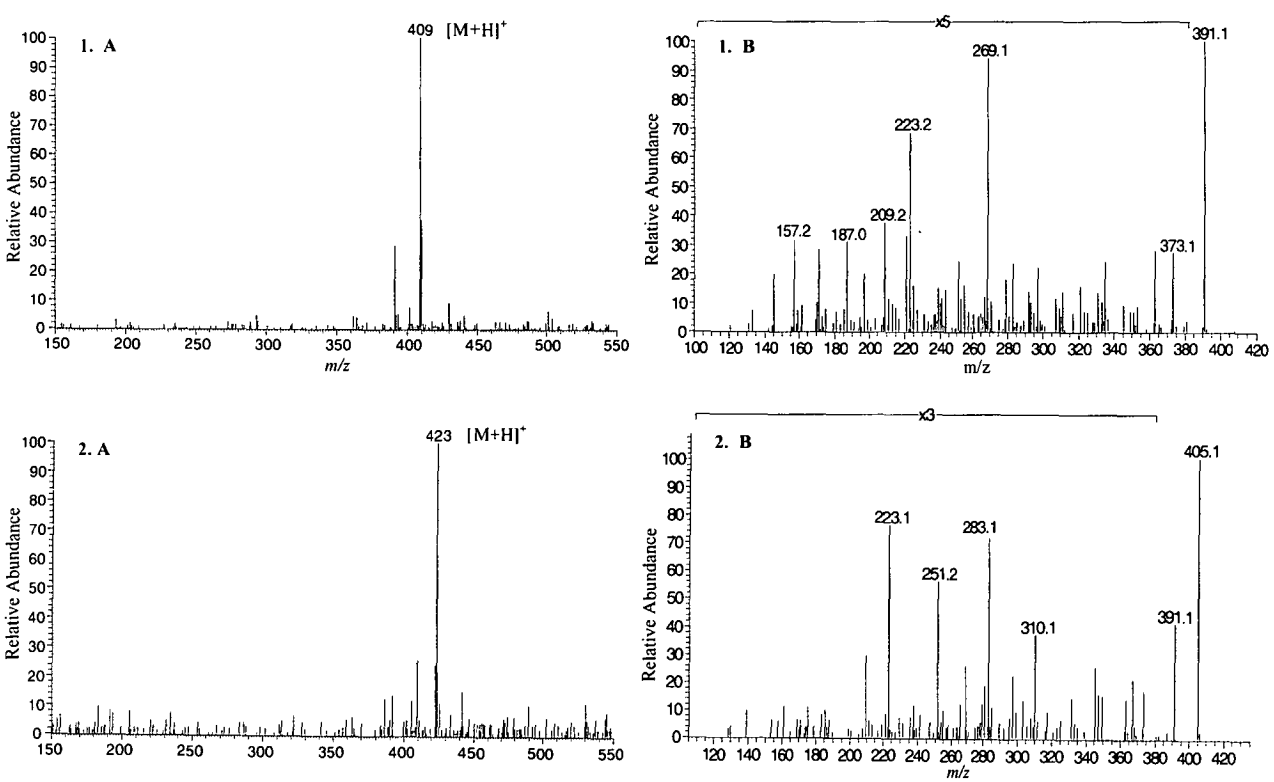


Figure 2.1 UV-Vis spectra for five components of boronia flowers obtained using HPLC program C.

the data published in the aforementioned study. Given the relatively high level of C-13 norisoprenoids and the accepted biosynthetic link between C-40 carotenoids and norisoprenoids in other plants the presence of C-27 apocarotenoids in boronia might be expected.

APCI mass spectrometric data were then obtained from each of the five HPLC peaks (assigned as peaks **1** through to **5**) and used to propose identities for these apocarotenoids. **Figure 2.2** gives the MS data (**A**) and MS/MS data (**B**) respectively. The molecular weights obtained from the APCI mass spectra of peaks **3** and **4** corresponded to known C-27 apocarotenoids (Straub, 1987). The APCI mass spectrum for peak **3** was consistent with both β-apo-10′-carotenoic acid and a hydroxy-β-apo-10′-carotenal ($C_{27}H_{36}0_2$ / $[M+H]^+$ = 393). The spectrum for peak **4** was consistent with β-apo-10′-carotenal ($C_{27}H_{36}0$ / $[M+H]^+$ = 377). For further clarification, flower extract was methylated with diazomethane and analysed in comparison to an unmethylated extract using HPLC program A (**Figure 2.3**). Peaks **1** and **3** were removed by methylation indicating the presence of a carboxylic acid functional group. The resulting methyl esters appeared at the same retention time and

Figure 2.2 APCI mass spectra (A) and MS/MS spectra (B) for components 1 through 5.



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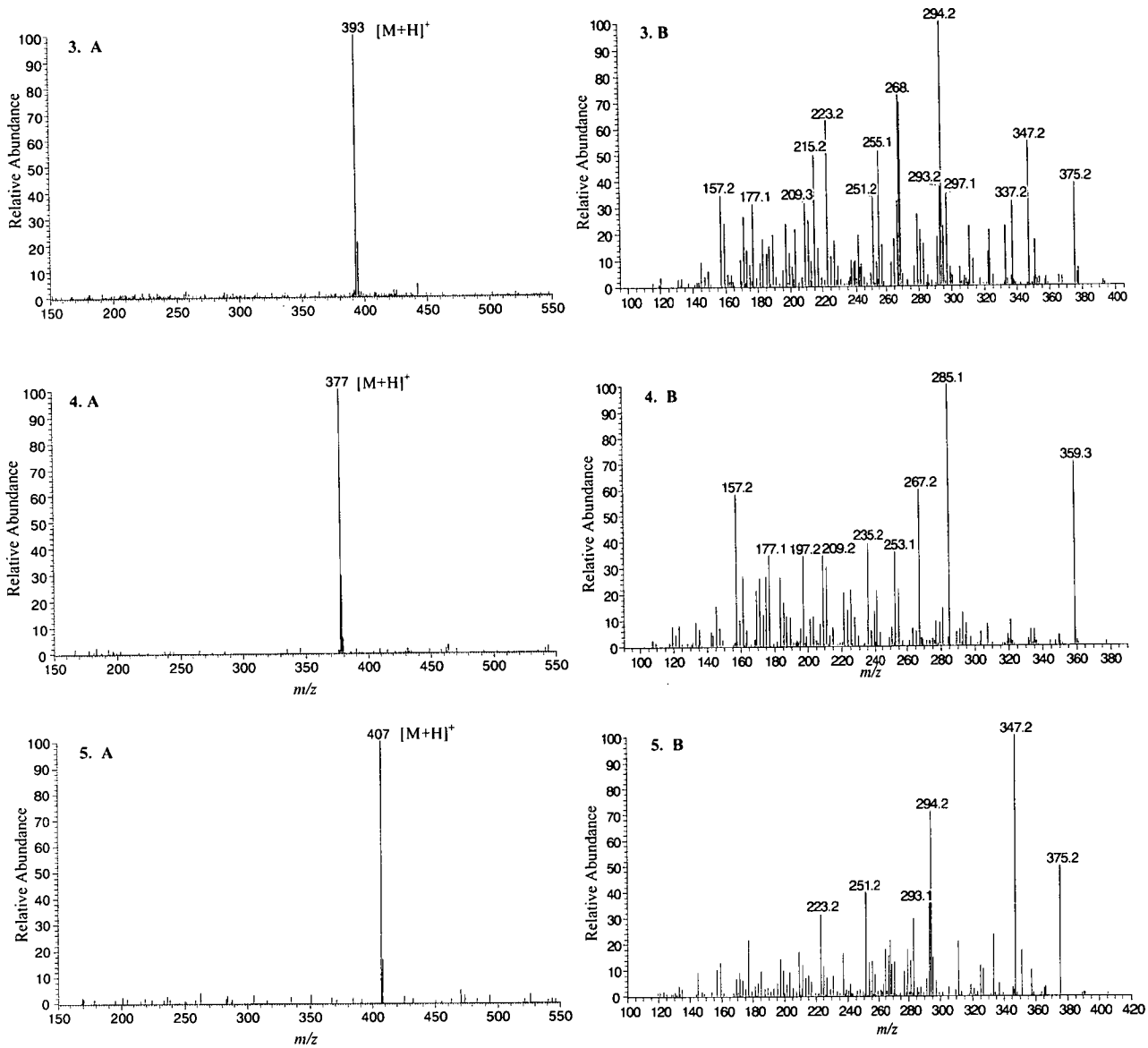


with the same APCI MS data as components **2** and **5**, augmenting the existing peak areas relative to that of lutein. Peak **4** was not affected by methylation with diazomethane, consistent when the UV-Vis and molecular weight data were considered, with an apo-10′-carotenal. The methylation experiment demonstrated peak **3** to be a carboxylic acid which, when considered in conjunction with UV-Vis and molecular weight data was consistent with an apo-10′-carotenoic acid. Peak **5**, based on its later retention time and its exact match in HPLC, UV and MS data to the product of methylation of peak **3**, was proposed as methyl apo-10′-carotenoate. Similarly, peaks **1** and **2** were consistent with a hydroxy-apo-10′-carotenoic acid and a methyl hydroxy-apo-10′-carotenoate, respectively.

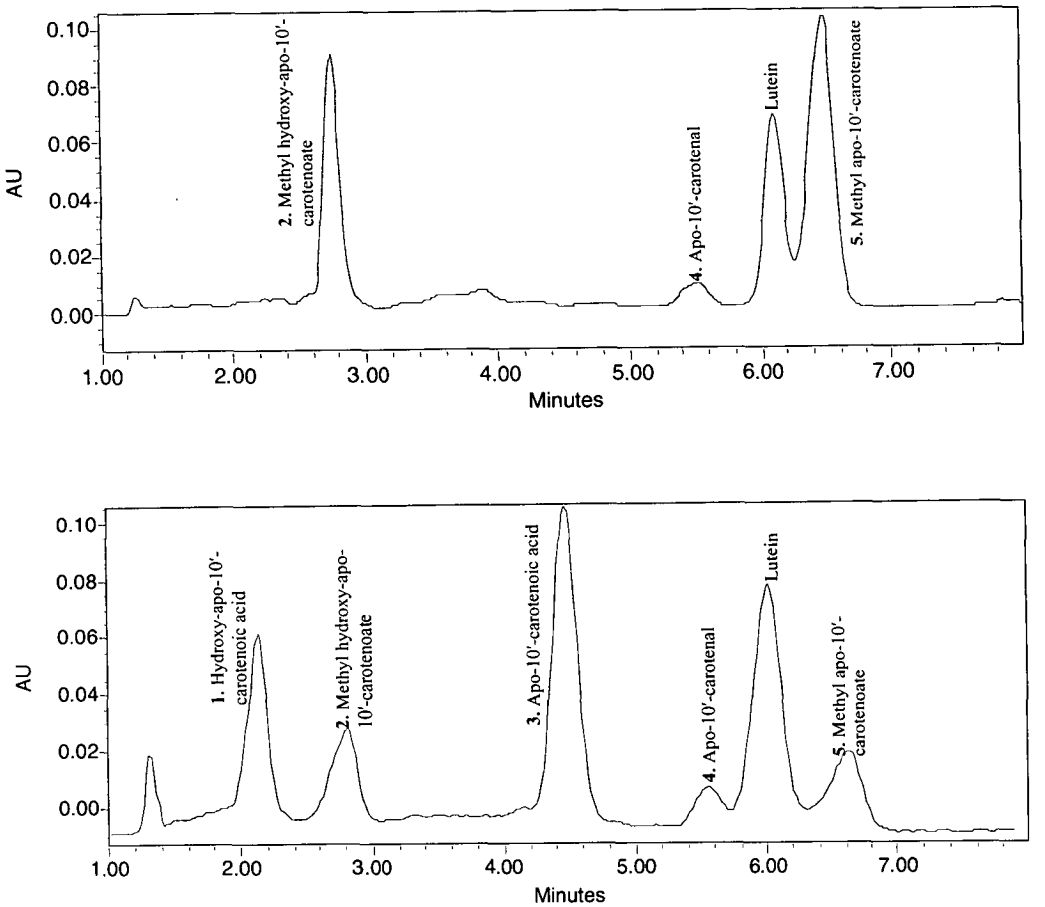


Figure 2.3 Change in the HPLC (Program A) apocarotenoid profile following methylation with diazomethane at 430 nm. The bottom chromatogram is the unmethylated sample.

The MS/MS data detailed in **Figure 2.2** further supported the proposed identities. The five peaks gave the relatively complex product ion profiles typical of carotenoids (van Breeman *et al.*, 1996). These included characteristic ions such as the loss of toluene (eg. the base peak for compound **4**). In addition, specific functional group losses from the apocarotenoids supported the proposed structures. Hydroxy-apo-10'-carotenoic acid (**1. B, Figure 2.2**, $[M+H]^+$ at *m/z* 409) showed successive loss of water from the hydroxyl and carboxyl groups respectively (peaks at *m/z* 391 and *m/z* 373). The peak at *m/z* 363 ($[M+H-46]^+$) was consistent with the loss of formic acid, which is typical of carboxylic acids. Methyl hydroxy-apo-10'-carotenoate (**2. B, Figure 2.2,** $[M+H]^+$ at *m/z* 423) showed losses of 18, 32, 50, and 60 daltons which were consistent with losses of water, methanol, methanol plus water, and methyl formate respectively. The MS / MS spectrum for apo-10'-

carotenoic acid (**3. B, Figure 2.2**, $[M+H]^+$at *m/z* 393) showed peaks for losses of water at *m/z* 375 and formic acid at *m/z* 347 from the hydroxyl and carboxyl groups respectively. The apo-10'-carotenal MS/MS spectrum (**4. B, Figure 2.2**, $[M+H]^+$ at *m/z* 377) showed a loss of only one water at *m/z* 359 as would be expected from an aldehyde. Peaks for losses of methanol at *m/z* 375 and methyl formate at *m/z* 347 were apparent in the MS/MS spectrum for methyl apo-10'-carotenoate (**5. B, Figure 2.2**, $[M+H]^+$ at *m/z* 407).

It should be noted that while the published data refers to the β isomers of these apocarotenoids, and there is no published data referring to an α isomer, the data obtained in this study cannot readily distinguish between α and β-isomers. However, the presence of an ion at $[M+H-56]^+$ in compounds **3**, **4** and **5** could be interpreted as characteristic of retro Diels-Alder fragmentation from an ε carotenoid ring (Van Breeman *et al.*, 1996). Similarly, our data does not allow the cis/trans geometry or the position of the hydroxyl moiety to be established with any certainty. Furthermore, close scrutiny of the mass chromatograms for apo-10'-carotenoic acid, apo-10'-carotenal and methyl apo-10'-carotenoate (**Figure 2.4**) clearly showed evidence of two or more unresolved isomers of each compound.

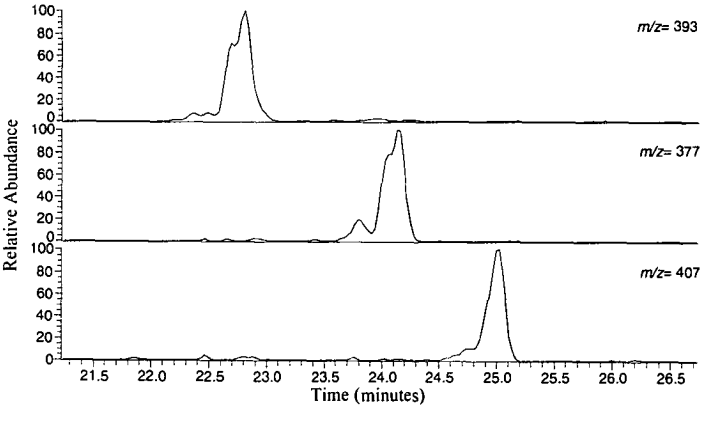


Figure 2.4 Mass chromatograms, obtained using analytical program C, of apo-10'-carotenoic acid (*m/z* 393), apo-10'-carotenal (*m/z* 377), and methyl apo-10'-carotenoate (*m/z* 407) showing the presence of multiple isomers.

However, the protonated molecules, $[M+H]^+$, obtained in each mass spectrum and the MS/MS spectra for the five apocarotenoids are in agreement with the proposed identities. In addition, the extracted mass chromatograms align with the corresponding HPLC positions (**Figure 2.5**).

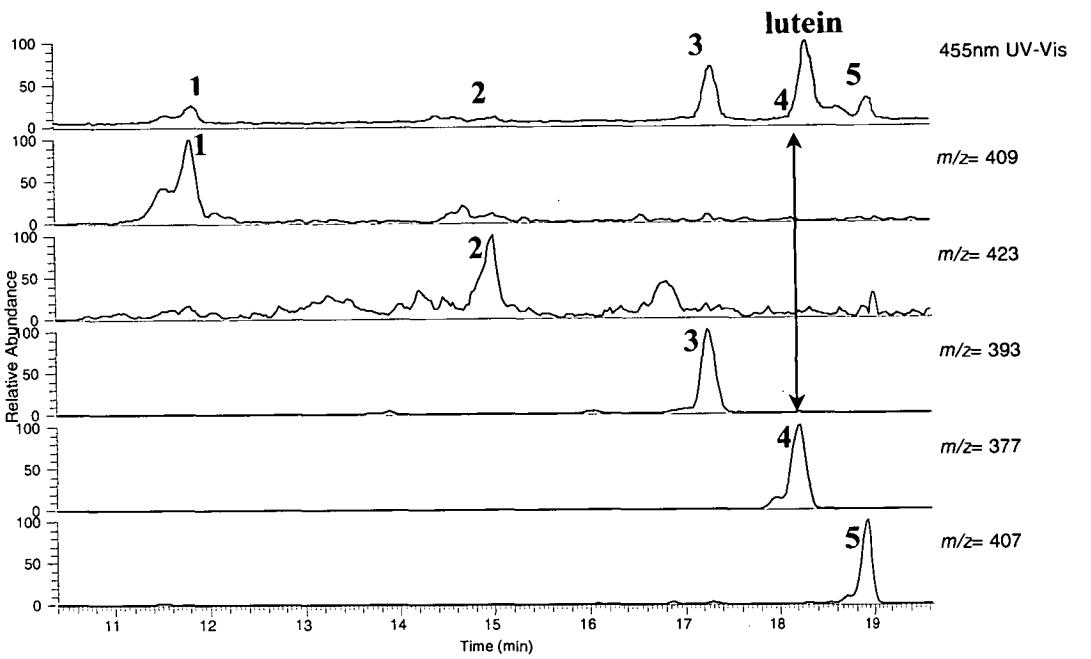


Figure 2.5 Individual mass chromatograms for each of the apocarotenoids showing alignment with their corresponding HPLC chromatogram positions. Component **4** (apo-10'-carotenal) is an unresolved shoulder slightly to the left of lutein. Separation was achieved using the HPLC program B.

Structures of possible 'all trans', β, and 3-hydroxy isomers of the apocarotenoids are presented in **Figure 2.6**. Both β-apo-10'-carotenoic acid (**3, Figure 2.6**) and β-apo-10'-carotenal (**4, Figure 2.6**) have been reported in the literature. The former has been isolated only from animal sources (Al Hasani and Parrish, 1972; Sharma *et al.*, 1977) while the latter has been previously identified in other plant species. These reports include identification of β-apo-10'-carotenal in three varieties of Citrus *senensis* (Gross *et al.*, 1972), lichens from Kenya (Czeczuga *et al.*, 1992) and the Brazilian fruit Mammea americana (Godoy and Rodríguez-Amaya, 1994). The related compound 3-hydroxy-β-apo-10'-carotenal, not identified in this study, has been found in the flavedo of sinton citrangequat (Yokoyama and White, 1966). In this study one or more isomers of the four C-27 apocarotenoids, hydroxy-apo-10'-

carotenoic acid, methyl hydroxy-apo-10′-carotenoate, apo-10′-carotenoic acid, and methyl apo-10′-carotenoic acid are reported here as being derived from plant sources for the first time.

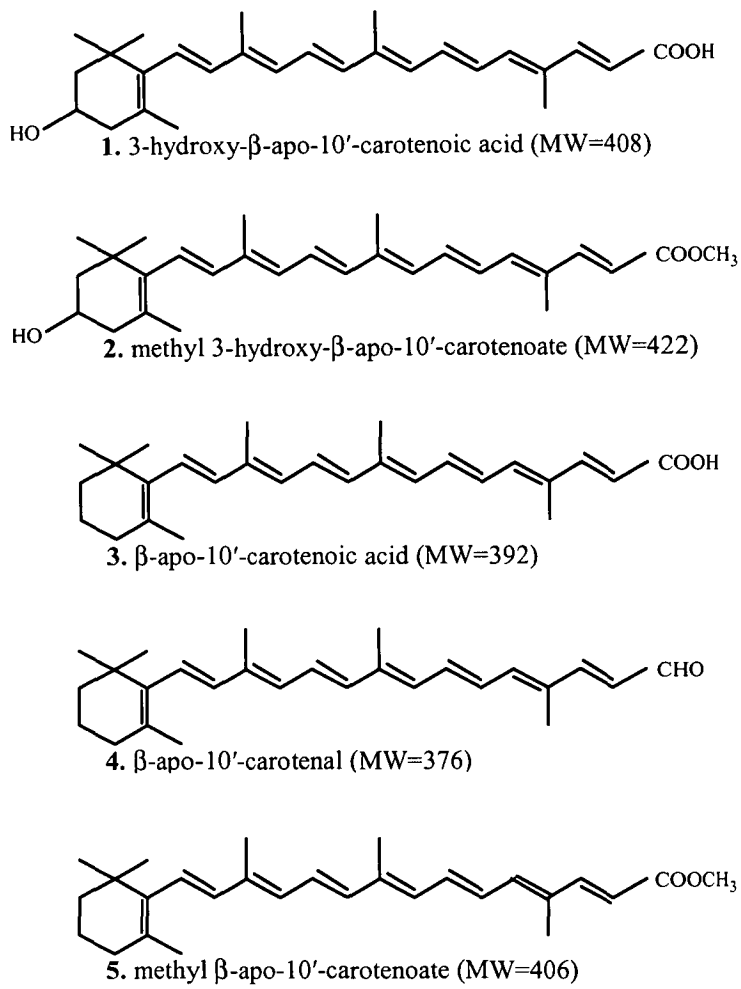


Figure 2.6 Possible structural formula of the five apocarotenoids identified in boronia flowers.

The C-27 apocarotenoids detected here and the C-13 norisoprenoids previously characterized (Weyerstahl *et al.*, 1995) are matched in both chain length and structure to known longer chain carotenoids. The structural matching of these compounds is evidence that C-13 norisoprenoids are derived from C-40 carotenoids in boronia. Speculation as to a possible C-40 carotenoid parent molecule for β-ionone is important because of its relevance to the aroma profile of boronia extracts.

Based on the structures of the five C-27 compounds, five known carotenoids as published in Straub (1987) are possible parent compounds. They are β-carotene (β,β-carotene), α-carotene (β,ε-carotene), isocryptoxanthin (β,β-caroten-4-ol), β-cryptoxanthin (β,β-caroten-3-ol), and β,β-caroten-2-ol. Additionally, selection of clones with different carotenoid profiles may lead to the production of extracts with varying aroma characteristics, which may in turn increase market differentiation for Tasmanian boronia. These results should serve to provide the basis for future studies on the possible mechanism of C-13 norisoprenoid biosynthesis.

Chapter 3

Changes in Carotenoids and Carotenoid Cleavage Products during Flower Development

3.1 Introduction

The identification of C-27 apocarotenoids in boronia provides an opportunity to further explore the relevance of the biosynthesis of carotenoid derived flavour and aroma compounds to the yields of these compounds from boronia. Previous studies by MacTavish (1995) examined changes in extract yield, the total volatiles as a percentage of the extract, and changes in particular volatiles over 5 different stages of flower development. In that thesis MacTavish (1995) reported that the yield of extract, percentage of volatiles and β-ionone levels approximately doubled between the large bud and open flower stages. Additionally, MacTavish (1995) showed that there was variation in the appearance of different volatiles during flower development with β-ionone not appearing until the large bud stage. Measurement of the changes in C-27 apocarotenoids in relation to β-carotene and β-ionone levels during flower development may provide further insight into the metabolic processes that are occurring during flower development.

3.2 Materials and Methods

3.2.1 Materials

ß-ionone (97.2% by GC) and ß-carotene standards were obtained from Sigma. All other reagents were analytical grade. HPLC grade solvents were used for chromatographic purposes.

3.2.2 Flower Development Stages

Flowers at four different stages of flower development were analysed for changes to carotenoid and β-ionone levels. The development stages were selected in order to maximise observations at the time of bud opening and **Figure 3.1** is a photographic representation of the different stages of flower development. The selection of these stages allowed a more detailed examination of changes, than was undertaken by MacTavish (1995), during flower opening by including the newly opened bud stage. Buds and flowers were first separated with a sieve and then further selected with forceps in order to eliminate any overlap between the stages. The large bud stage just prior to opening (Stage 2) in this study was equivalent to the large bud stage (stage 4) in the studies performed by MacTavish (1995).

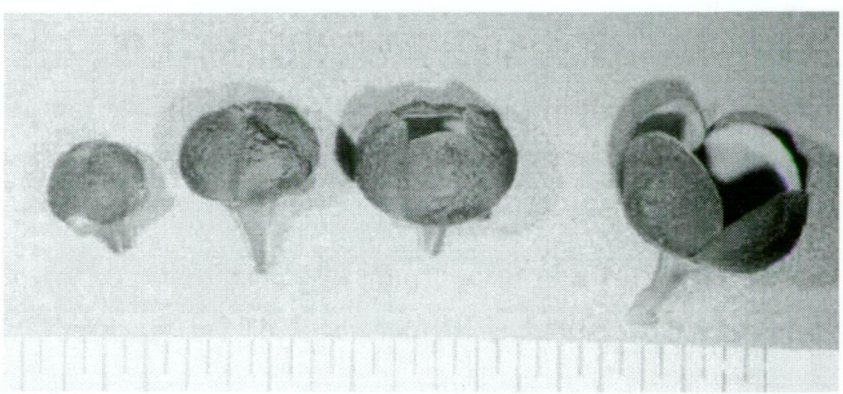


Figure 3.1 Photographic representation of the four different stages of boronia flower development selected for measuring quantitative changes in C-40 carotenoids and metabolites. (The divisions on the scale are 1 mm) Stage 1 is small buds. Stage 2 is large buds just prior to opening. Stage 3 is newly opening buds (1-3 mm open) whilst stage 4 is open flowers.

3.2.3 Carotenoid Extractions

A small scale extraction method, based on the extraction method described in chapter 2, was developed in order to study carotenoid and ß-ionone changes during flower development. Flowers or buds (200 – 300 mg) were homogenised using an ultraturrex (T25 basic, Ika labortechnik, setting 6), fitted with a 10 mm head, with

2.5 mL of acetone in the presence of 125 mg calcium carbonate. Following centrifugation at 10,000 g (Beckman J2 – 21 M / E, rotor 20.1) the pellet was extracted twice more with 2.5 mL of acetone and the supernatants combined. The combined acetone fractions were diluted to a total volume of 9 mL with acetone prior to analysis by HPLC and HPLC/MS. All operations were at 4°C under pale yellow or reduced light.

3.2.4 Analytical Techniques

Extracts were analysed using the HPLC and HPLC / MS methodology detailed in sections 2.2.3 and 2.2.4 respectively.

3.3 Results and Discussion

3.3.1 Identification of Boronia Carotenoids

The carotenoid profiles of boronia leaves and flowers were examined. Boronia leaves had a typical leaf carotenoid profile (Britton, 1998) with neoxanthin, violaxanthin, lutein and β-carotene present similarly in the leaves from two different boronia clones (3 and 250). Chromatograms showing the elution pattern of the leaf carotenoids are presented in **Figure 3.2**. The flowers however presented a much more complex pattern with significant changes occurring during flower development. **Figure 3.3** shows chromatograms (extracted at 430 nm) of the carotenoid profiles at four different stages of development in the flowers of clone 250 boronia. In addition to the C-27 apocarotenoids discussed in chapter 2, analysis of the profile of open boronia flowers allowed positive identification of the C-40 carotenoids β-carotene, zeaxanthin, lutein and neoxanthin. This contrasted with the leaf profile in that violaxanthin was absent in the flowers and zeaxanthin was absent in the leaves.

The C-40 carotenoids in both flowers and leaves were identified on the basis of known standards and UV-Vis spectra which are presented in **figure 3.4**. In addition to the C-40 carotenoids positively identified in the flowers a number of carotenoid peaks were either too small to provide any analytical data or they allowed a partial characterisation. The UV-Vis spectra for four unknown carotenoids (see **Figure 3.5**) were extracted from the data. Three of the carotenoids (A, B and D) had similar spectra consistent with these compounds having the same chromophore (Liaaen-Jensen, 1995). Furthermore, carotenoid D had a double cis peak at 297 nm indicating that it contained a cis-isomer within the chromophore structure (Liaaen-Jensen, 1995). The elution position and UV-Vis spectra of carotenoid D (in the hydrocarbon region) was consistent with it being a ζ-carotene isomer (for UV-Vis spectra see pp. 37-39 and 41, Britton *et al.*, 2003). Interestingly it has been noted that ζ-carotene accumulated in the roots of *Zea mays* and *Medicago truncatula* in association with the arbuscular mycorrhizal correlated increases in C-13 norisoprenoids (Fester *et al.*,

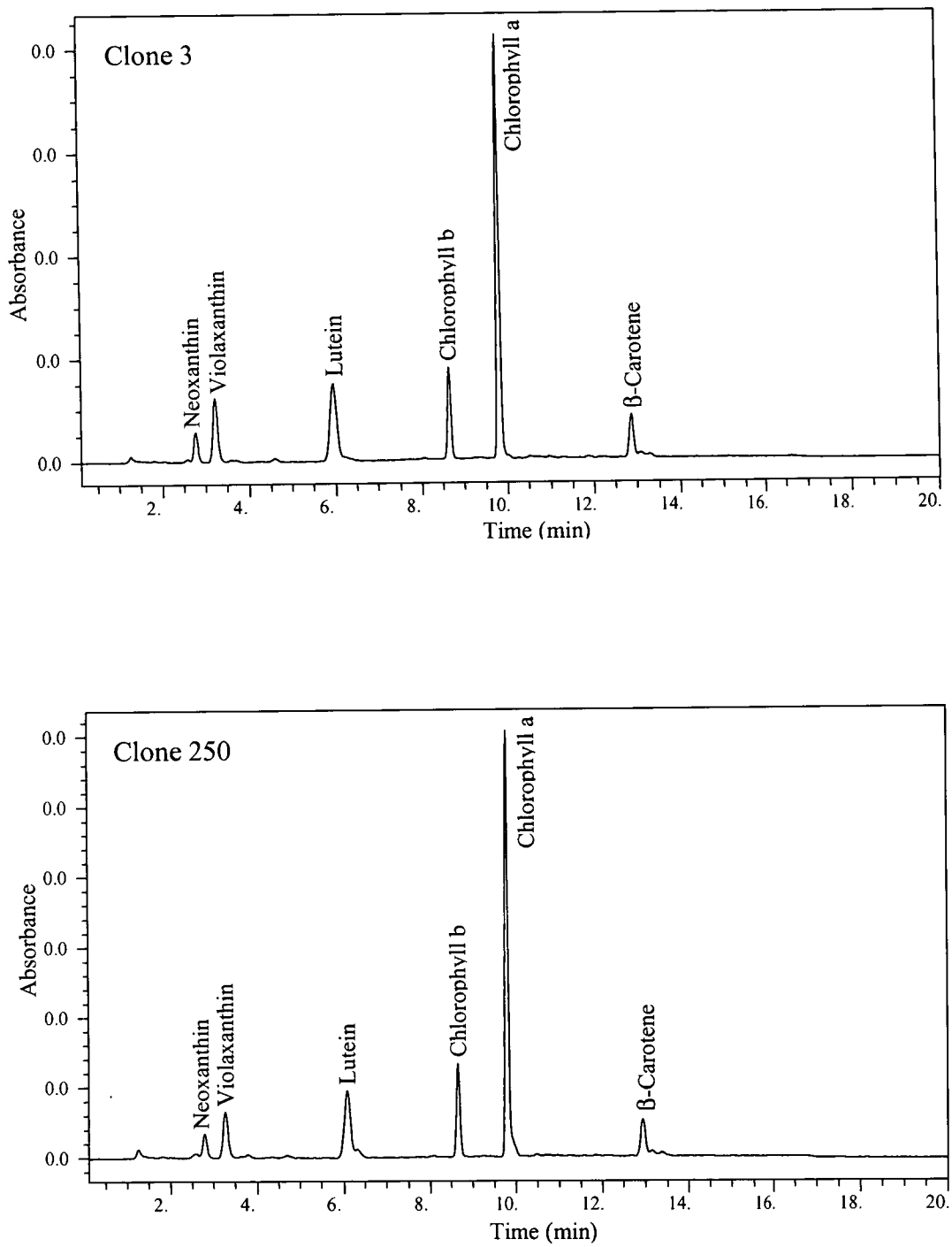


Figure 3.2 Carotenoid profiles of boronia leaf for clones 3 and 250.

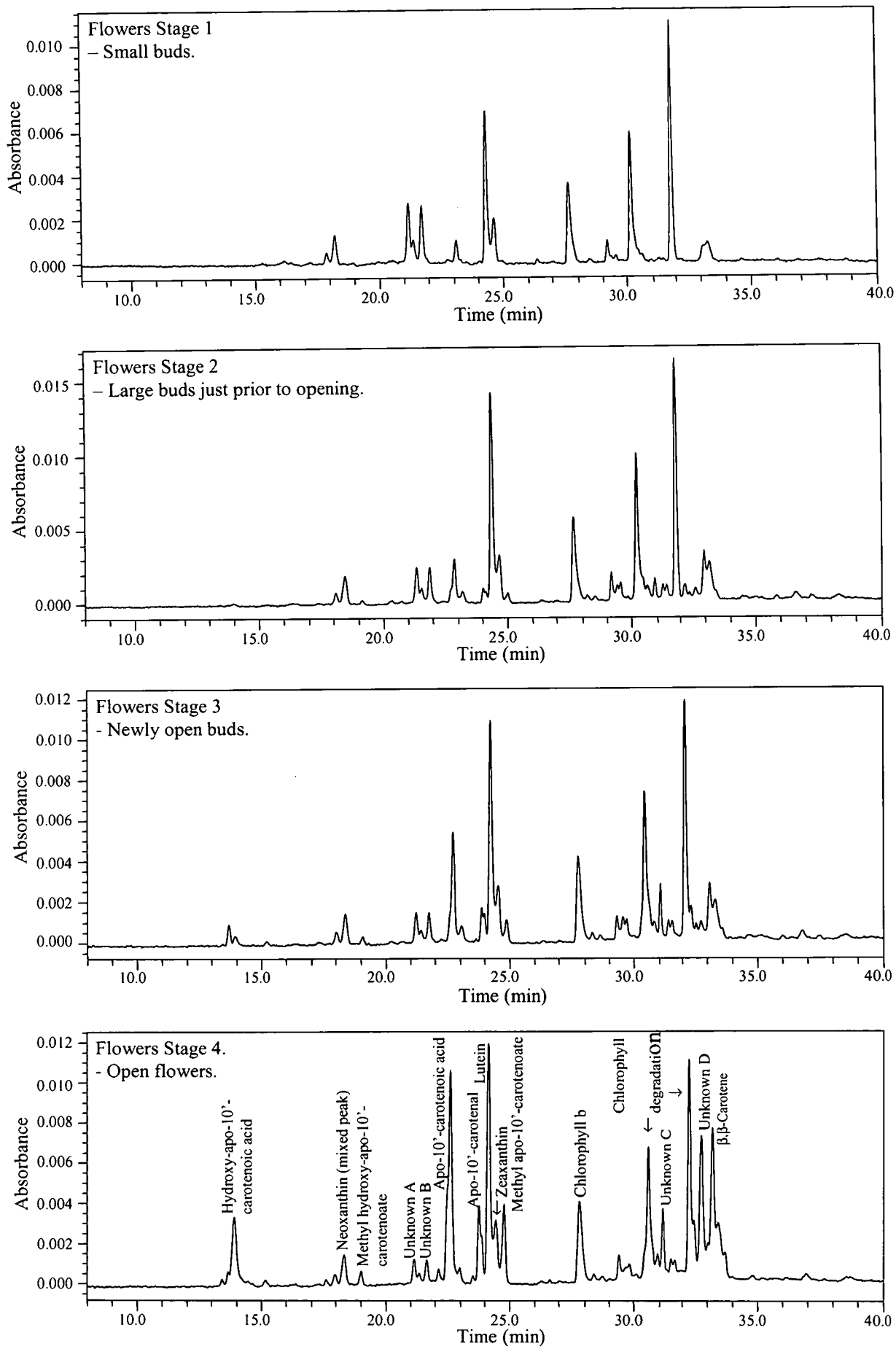


Figure 3.3 Changes in boronia pigments at different stages of flower development.

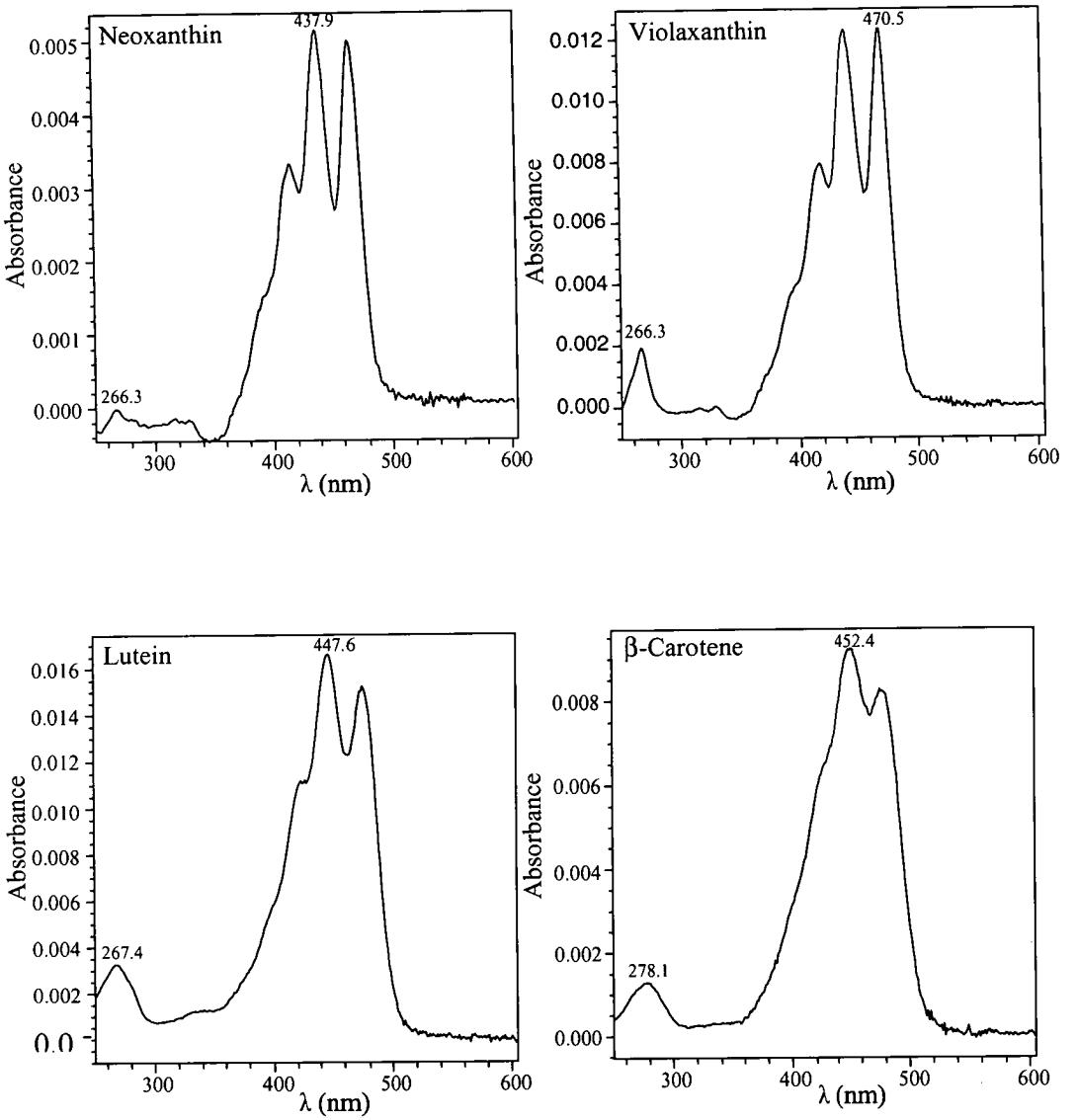


Figure 3.4 Extracted UV – Vis spectra of positively identified C-40 carotenoids in boronia.

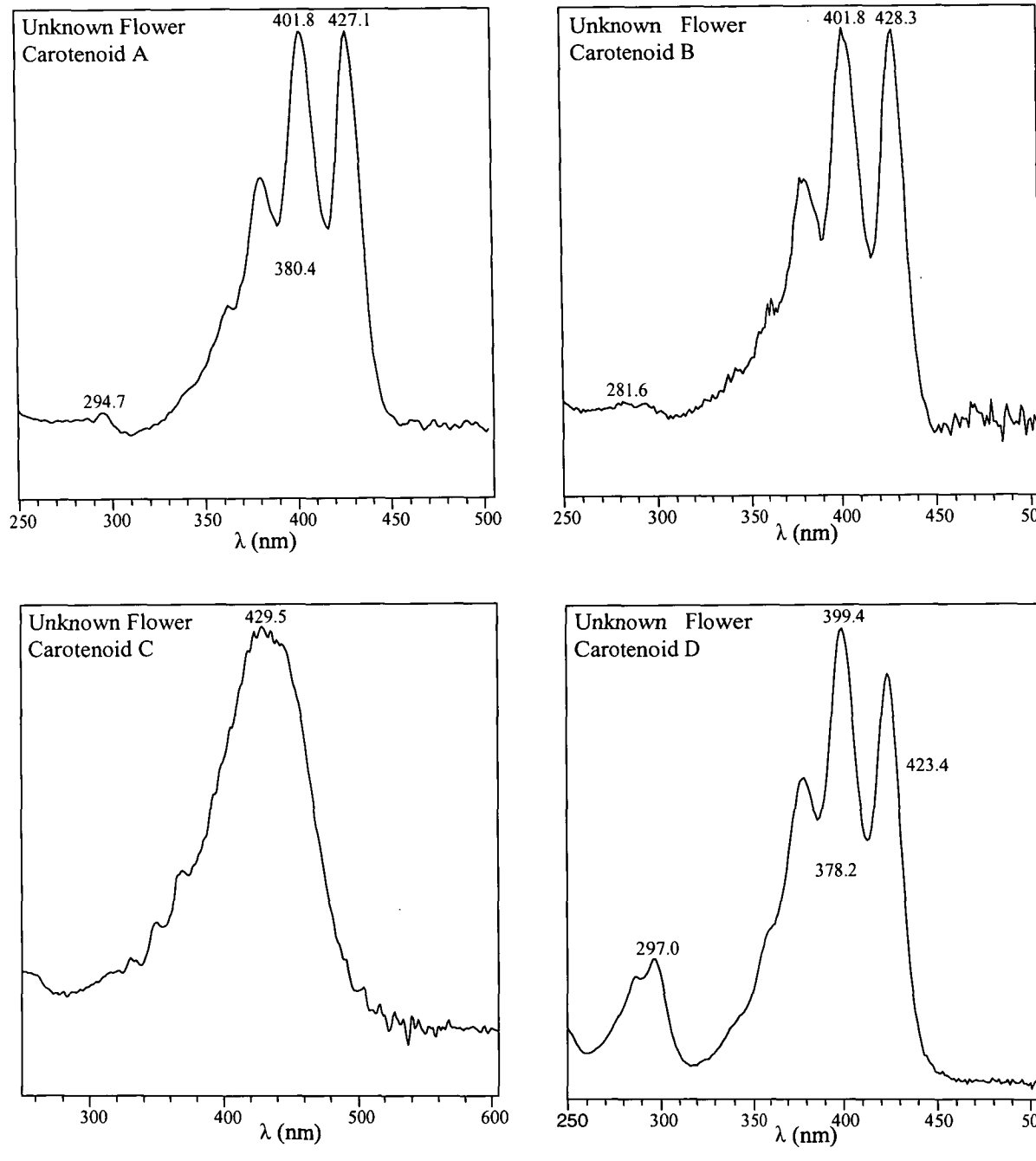
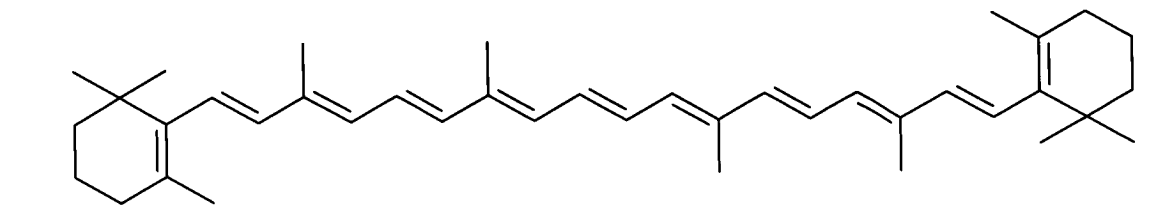


Figure 3.5 UV-Vis spectra for four unknown carotenoids in boronia flowers.

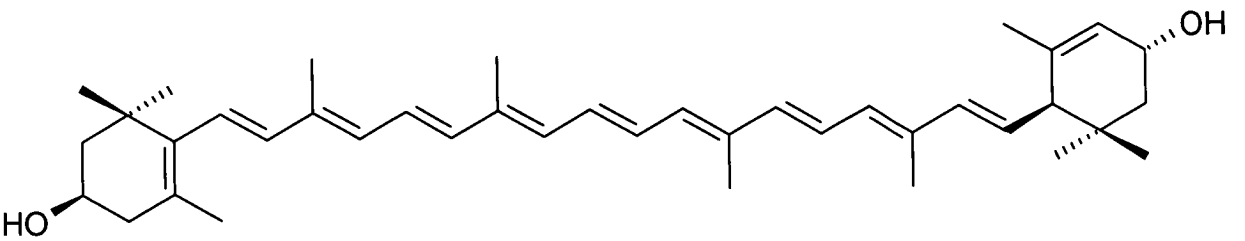
2002). Whilst more work needs to be done with regard to the identification of carotenoid D it may be that an increase in ζ-carotene is associated with an increase in carotenoid biosynthesis in more than one plant species.

The UV-Vis spectrum for Compound C was a broad single peak with an absorption maxima at 430 nm consistent with it being a C-27 apo-carotenoid. The same compound was found to be present in boronia absolute and a mass spectrometric analysis (see **Appendix 2**) indicated the compound had a molecular weight of 646. This allowed the compound to be tentatively assigned as the palmitic acid ester of hydroxy apo-10'-carotenoic acid. Analysis of the MS/MS spectrum gave supporting information with the peak at *m/z* 391 corresponding to the loss of palmitic acid. Esters of palmitic acid are commonly found in plants and Straub (1987) refers to di-palmitate esters of zeaxanthin, lutein, taraxanthin, and astacene. Palmitic acid was one of the main fatty acids bound to xanthophylls in potatoes (Breithaupt and Bamedi, 2002), β-cryptoxanthin in various fruits and vegetables (Breithaupt and Bamedi, 2001), and to xanthophylls in fruit extracts (Khachik *et al.*, 1989). Additionally those studies (Khachik *et al.*, 1989; Breithaupt and Bamedi, 2001) have demonstrated that fatty acid esters of carotenoids elute at a later time on C-18 columns than the parent molecule. This pattern is also observed in boronia further supporting the proposed assignment. Whilst there is no reported evidence in boronia of C-40 carotenoid esters this may be the first example of a palmitic acid ester of an apocarotenoid occurring in plants.

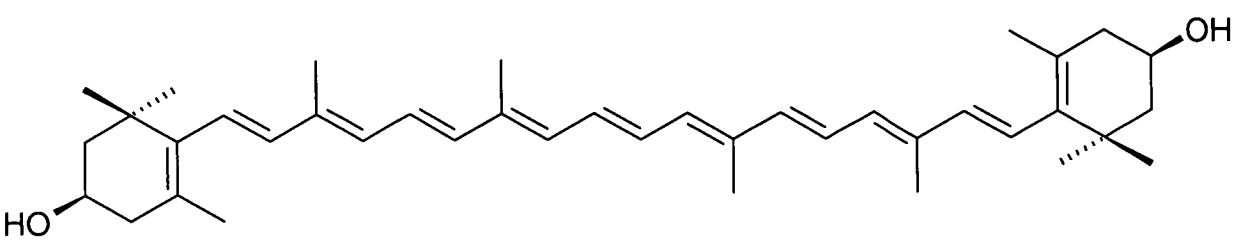
The structures of the C-40 carotenoids found in boronia allowed an assessment of their potential role as precursors of the C-13 norisoprenoids identified in boronia (Davies and Menary, 1983; Weyerstahl *et al.*, 1994; Weyerstahl *et al.*, 1995). These carotenoid structures are presented in **figure 3.6.** A comparative analysis of these structures with those of known C-13 norisoprenoids (see **figure 1.4)** and the C-27 apocarotenoid identified in boronia flowers (see **figure 2.6)** allowed some insight with regard to carotenoid cleavage in boronia. Cleavage products of β-carotene and zeaxanthin that would result in the biosynthesis of compounds positively identified in boronia are outlined in **figure 3.7.** Whilst the matching apocarotenoid fragment of zeaxanthin was a carotenoic acid, oxidative cleavage of parent carotenoids was



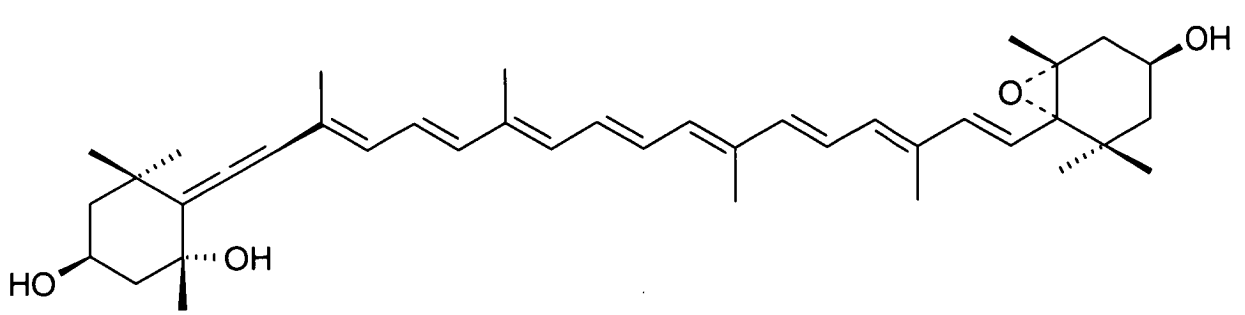
β-carotene



Lutein



Zeaxanthin



Neoxanthin

Figure 3.6 The structure of C-40 carotenoids found in boronia flowers.

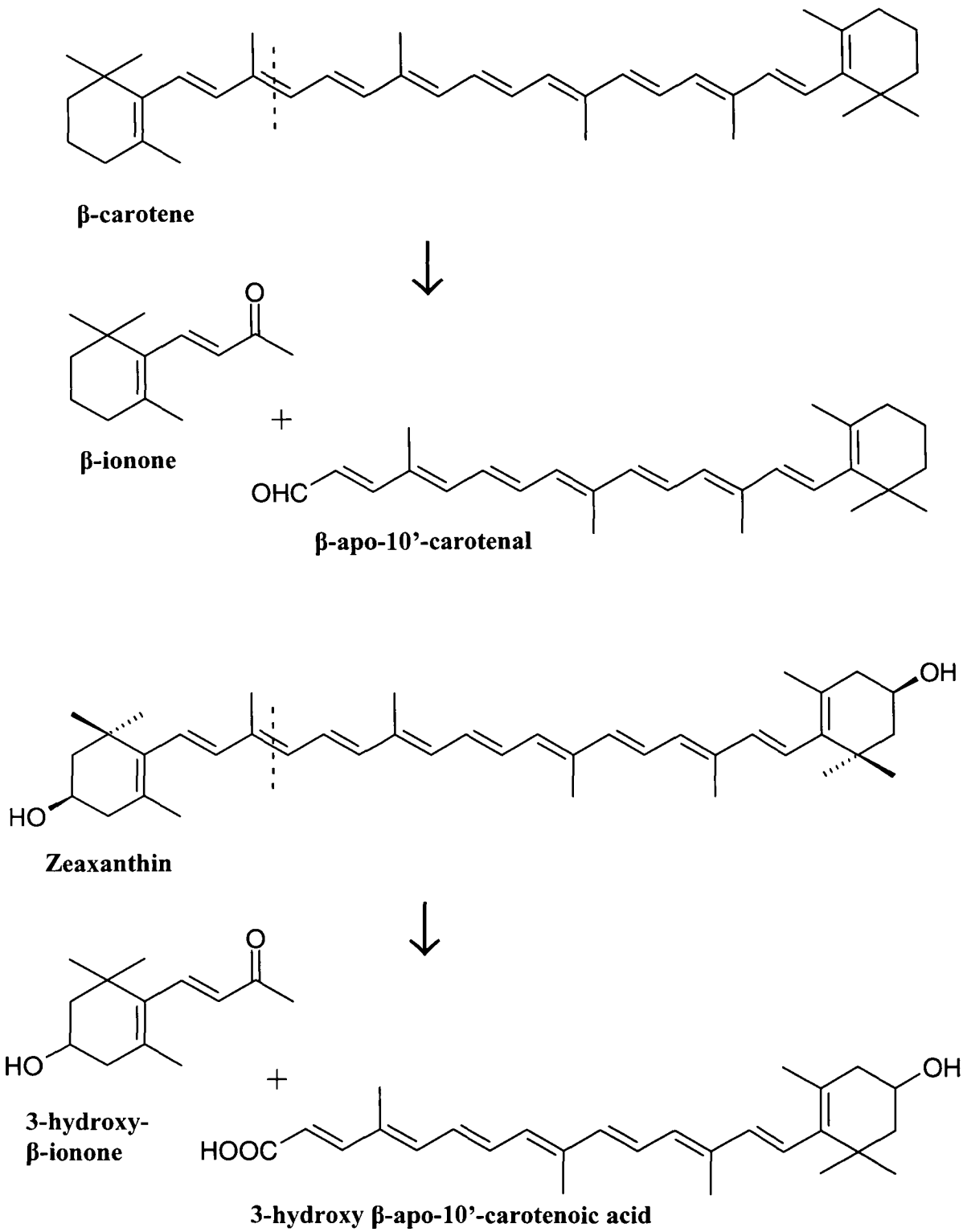


Figure 3.7 Carotenoid cleavage rationales in boronia flowers.

expected to result in the formation initially of either aldehydes or ketones with further oxidation leading to the formation of carboxylic acids or alcohols (Knapp *et al.*, 2002). The 3-hydroxy apo-10'-carotenal was not detected in boronia.

It should be noted that all of the C-27 apocarotenoids detected in boronia are structurally matched to either β-carotene or zeaxanthin. However cleavage of other identified carotenoids has the capacity to produce several of the C-13 norisoprenoids that have been identified in boronia (Weyerstahl *et al.*, 1995). Cleavage of lutein has the capacity to produce both α-ionone and the α isomers of the C-27 apocarotenoids. Further consideration identified neoxanthin as a possible precursor of 5,6-epoxy-β-ionone through their matching end group structures. This compound would result from cleavage in the 9', 10' position. However it should be noted that the grass hopper ketone, which is the 9, 10 cleavage product of neoxanthin and a β-damascenone precursor (Suzuki *et al.*, 2002), has not been detected in boronia.

The degradation pathways proposed for boronia have been similarly discussed in other studies including with regard to saffron, gardenia fruits, and apple leaves (Knapp *et al.*, 2002); watermelons and tomato (Lewinsohn *et al.*, 2005), and rose flowers (Suzuki *et al.*, 2002). The absence of violaxanthin (see **Figure 1.1** for structure) in flowers was consistent with the observation that there have been no C-13 norisoprenoids identified from boronia flowers that would be expected on a structural basis to be derived from this xanthophyll. Additionally, the absence of violaxanthin cleavage products in boronia flowers does not support the rationale that aroma compounds are synthesised in the leaves of plants and transported to the flowers as glycosidic conjugates before release through glycosidase action (Winterhalter and Skouroumounis, 1997), as violaxanthin was found in boronia leaves.

In boronia many of the ionols and ionones that are oxygenated in the 3 position on the ionone ring are readily matched in structural terms with lutein and zeaxanthin. Consideration was also given to possible precursors of the C-13 norisoprenoids identified in boronia which are oxygenated in the 4 position. Whilst no 4-hydroxy xanthophylls have been identified in boronia there were a number of unidentified carotenoids with two compounds in the xanthophyll region of the chromatogram

(unknown carotenoids A and B). Future work in this area should consider the structure of the unknown carotenoids in order to further understand their possible roles as structural precursors of the 4-hydroxy ionones.

3.3.2 Changes during Flower Development

Where possible, given the complexity of the chromatograms, the changes in C-40 carotenoids, C-27 apo-carotenoids, β-ionone, and some unknown carotenoids were then measured quantitatively in the flowers from both clones 3 and 250. β-carotene and β-ionone were measured quantitatively with known standards, whilst lutein was measured by relating the β-carotene and lutein extinction coefficients. The other carotenoids and apo-carotenoid levels are expressed as relative to β-carotene through using the extinction coefficient for β-carotene in calculations.

Measurement of β-carotene levels (**Figure 3.8**) during flower development showed that this compound was present in small buds (stage 1). Small changes were then apparent with a marked increase occurring in development from the just opened bud stage to the open flower stage. These changes contrasted with the situation that was apparent for lutein (**Figure 3.9**) where there was an increase during bud development (between stages 1 and 2) with a constant level maintained during the later stages of flower development. Similarly, the data for the other positively identified xanthophylls, neoxanthin and zeaxanthin (not presented graphically), indicated that the levels of these compounds did not change during flower development.

The concentration of β-carotene in the open flower stage ranged from 42 mg / kg in clone 3 flowers to 12 mg / kg in clone 250 flowers. The levels in open flowers are within the range previously reported in the literature for other plant species. Müller (1997), using HPLC and photodiode array detection, analysed the carotenoid levels in several plants and plant parts. The β-carotene levels reported in that study ranged from 90 mg / kg in carrots to 0.01 mg / kg in cauliflower. The lutein level (see **Figure 3.9**) for clone 3 was 53 mg / kg in open flowers and 47 mg / kg in open clone 250 flowers. Again these levels compared with other plant species. The highest

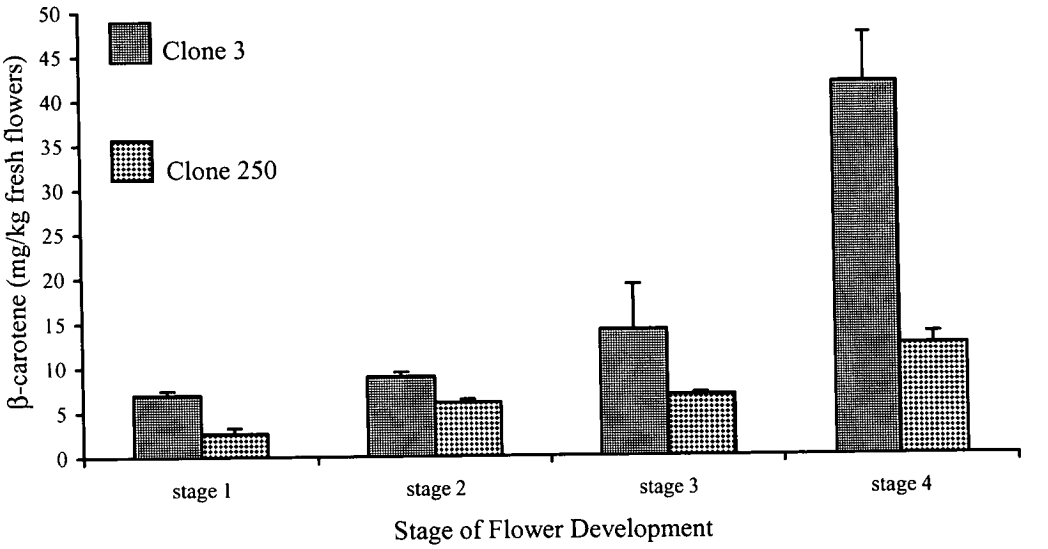


Figure 3.8 Changes in β-carotene during flower development of boronia clones 3 and 250.

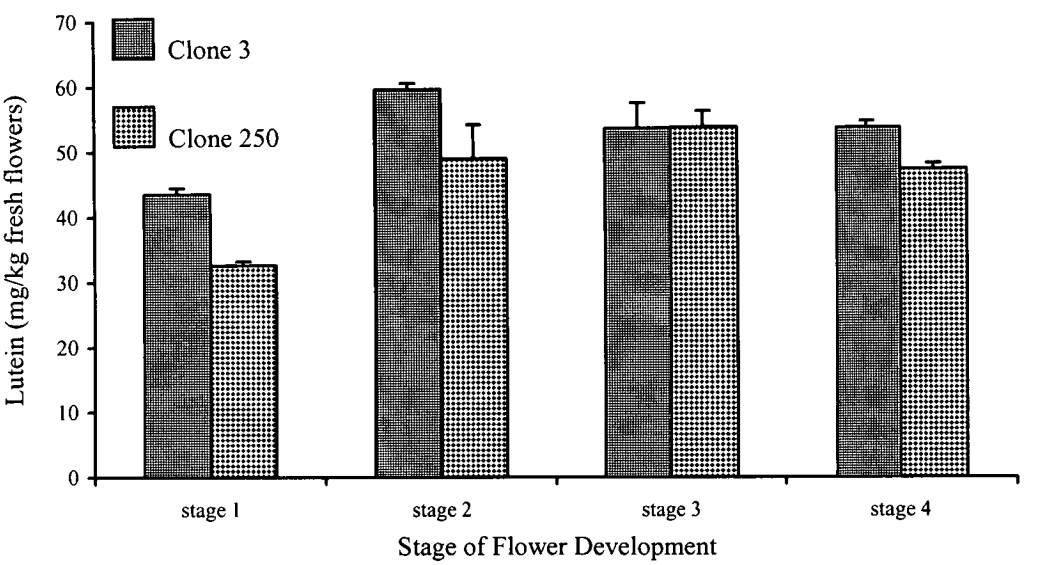


Figure 3.9 Changes in lutein levels during flower development in boronia clones 3 and 250.

lutein level reported by Müller (1997) was 186 mg / kg in kale with a large diversity of concentrations in other species.

The changes in β-carotene levels during flower development in boronia are similar in scale to the change in carotenoid levels observed during tomato fruit ripening. In tomatoes, carotenoids (mainly lycopene) increased by a factor of 10 to 14 (Bramley, 2002) and the authors noted that the initiation of lycopene biosynthesis is paralleled by changes in the organoleptic properties of the fruit. Tomato carotenoids play a role as precursors to important aroma compounds including β-ionone, which could be derived from β-carotene cleavage, and 3-hydroxy-β-ionone which could be derived from zeaxanthin or lutein cleavage (Simkin *et al.*, 2004). Tomatoes also contain high levels of geranyl acetone (Simkin *et al.*, 2004), an acyclic C-13 norisoprenoid thought to be derived from lycopene, suggestive that the volatile profile of C-13 norisoprenoids in this plant reflect the overall carotenoid makeup.

In boronia, β-ionone levels (see **Figure 3.10**) reached a maximum in opening flowers (stage 3) with the levels reducing to less than half in the open flowers (stage 4). This contrasted with the large increases in β-carotene in open flowers (stage 4). A possible reason for this is that the β-ionone was released from the flowers soon after the initiation of flower opening. Given that boronia flowers are routinely harvested at

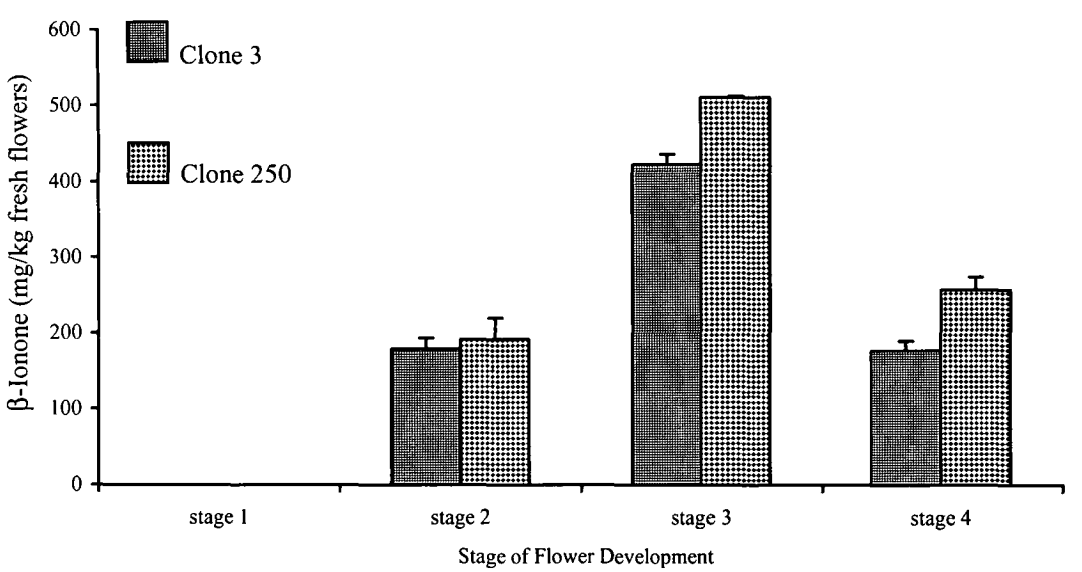


Figure 3.10 Changes in β-Ionone levels during flower development in boronia clones 3 and 250.

70-80% open flowers it is apparent that most of the β-ionone produced by the flowers is lost to the commercial process. Therefore harvest timing is likely to be critical to the yield of β-ionone. The rate of biosynthesis of plant volatiles versus the rate of release from the plant is a complex process with release dependent on several factors including the volatility of the particular compound and compartmentalisation factors relating to the site of biosynthesis and the mechanism of excretion (Dudareva *et al.*, 2004). Other factors relating to biosynthesis of volatiles include the down regulation of volatile biosynthesis following pollination which has been demonstrated in petunia and snapdragon flowers (Negre *et al.*, 2003).

Important changes in metabolite levels during the time of flower opening had been anticipated on the basis of the experimental observations of MacTavish (1995). The observations made by that author included:

1. The detection of oil glands per petal increased significantly from 130 to 185 between the large bud stage and open flowers. MacTavish (1995) noted that this was probably due to swelling rather than initiation of new oil glands.
2. There were large increases in both extract yield and the percentage of volatiles in the extract between the large bud stage and open flowers.
3. β-Ionone first appeared at the large bud stage followed by a large increase in the open flower stage.
4. Different patterns of volatile appearance were observed for β-pinene, limonene and heptadec-8-ene in comparison to β-ionone changes.

The data for changes in β-ionone levels found in this experiment contrasted with the data published by MacTavish (1995) whereby this study showed that β-ionone levels decreased between the newly opening buds and open flower stages. However the observation by MacTavish (1995) indicating that the percentage of β-ionone levels in the extract decreased in crops when the percentage of open flowers in the crop was over 70% is suggestive of β-ionone loss in the later stages of flower development. Additionally, MacTavish (1995) did not measure β-ionone levels at the newly opening bud stage. The data also contradicted that published by Bussell *et al.* (1995). Whilst these authors found the highest concentration of β-ionone in fully open flowers there are a number of important distinctions that need to be highlighted.

Bussell *et al.* (1995), as in the MacTavish study, did not measure changes during the critical period of initial flower opening and consequently the increases measured in this study would not have been observable in either of the 1995 studies. Additionally, Bussell *et al.* (1995) used ethanol as an extraction solvent which is in contrast to the usual method for extraction of volatiles from boronia. No data was presented by the authors with regard to the solubility of β-ionone in ethanol.

It was apparent from the data obtained in this study that carotenoid biosynthesis was ongoing at the time of flower opening and the data consideration of a rationale for the post harvest increases in β-ionone demonstrated by MacTavish and Menary (1998a). Ongoing carotenoid metabolism and site-specific cleavage in the 9,10 position may then have the potential to result in the large increases observed in incubated flowers after harvest. Related to this it is important to note that the level of β-carotene found in boronia does not stoichiometrically match the appearance of β-ionone. The data indicated a rapid onset of metabolism at the initiation of flower opening and that carotenoid biosynthesis and cleavage may be occurring concurrently.

This onset of metabolism is additionally apparent from the increasing complexity of the carotenoid profiles during flower development that was initiated just prior to bud opening. A range of unidentified compounds was also apparent and the largest increase occurred for the Unknown Carotenoid D (see **figure 3.11**) which eluted in

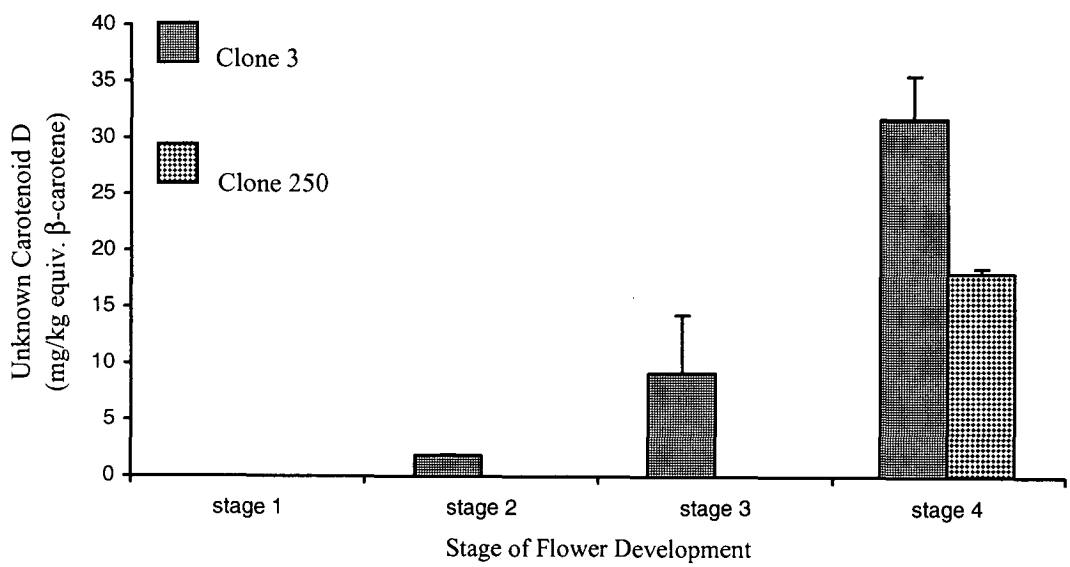


Figure 3.11 Changes in the levels of Unknown Carotenoid D (expressed as β-carotene equivalent) in boronia clones 3 and 250.

the hydrocarbon carotenoid section of the HPLC elution profile. In clone 3 flowers, this compound first appeared in large buds (stage 2) increasing to 31 mg / kg (β-carotene equivalent). A different pattern in clone 250 flowers occurred with this compound only present at the open flower stage. However the unknown carotenoids A and B (see **figure 3.12 and 3.13**) eluting in the xanthophyll region during HPLC and which were discussed in **section 3.3.1** decreased during flower development. This is different to the pattern of change observed for the other C-40 carotenoids and other unknown carotenoids and may reflect their role as precursor molecules. Identification of these unknowns will provide further insight into their precursor roles.

Changes in the relative levels of the C-27 apocarotenoids were measured where analytically possible and the results discussed here are based on β-carotene equivalence. The results showed similar patterns of increase with no apocarotenoids present in the small bud stage. Apo-10'-carotenoic acid (see **figure 3.14**) increased from 5.0 mg / kg in stage 2 buds to 9.8 mg / kg in newly opening buds with a three fold increase to 28.0 mg / kg. Levels of the related apo-10'-carotenal (**figure 3.15**) increased during flower development to 9.6 mg / kg in open flowers. Methyl apo-10'-carotenoate (**figure 3.16**) was found at similar levels in open flowers to the related aldehyde with clone 250 flowers reaching 8.1 mg / kg. The presence of methyl esters of the C-27 apocarotenoic acids is interesting and these particular apocarotenoids are not reported elsewhere. However methylation of carboxyl groups has been commonly observed in plants (Dudareva *et al.*, 2004).

The data for changes in hydroxy apo-10'-carotenoic acid levels during flower development of this compound is presented in **figure 3.17**. The data showed that small amounts were present in large buds with increases observed at the newly opening bud stage (stage 3). The largest increase occurred between newly opening buds (stage 3) and open flowers (stage 4) in both clones with levels reaching 14.6 mg / kg in clone 3 flowers. The increases in hydroxy apo-10'-carotenoic acid may be related to the cleavage of xanthophylls thus supporting the rationale that carotenoid cleavage operates on a range of carotenoids.

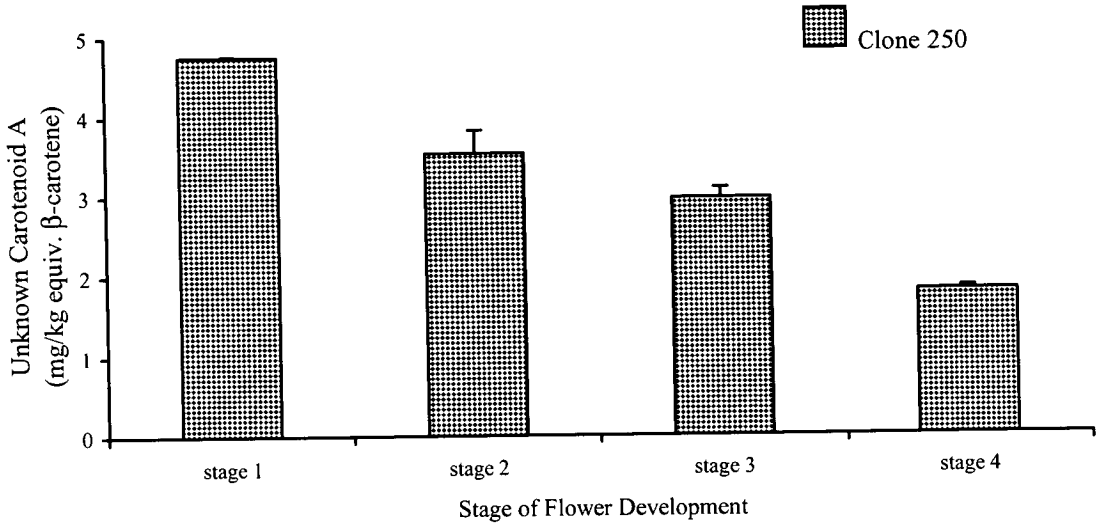


Figure 3.12 Changes in the levels of Unknown Carotenoid A (expressed as β-carotene equivalent) in boronia clone 250.

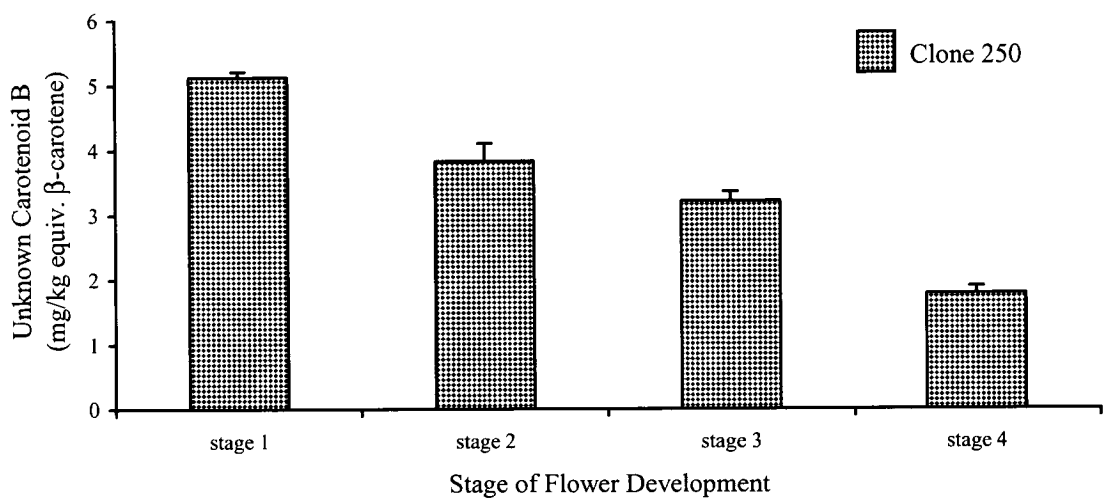


Figure 3.13 Changes in the levels of Unknown Carotenoid B (expressed as β-carotene equivalent) in boronia clone 250.

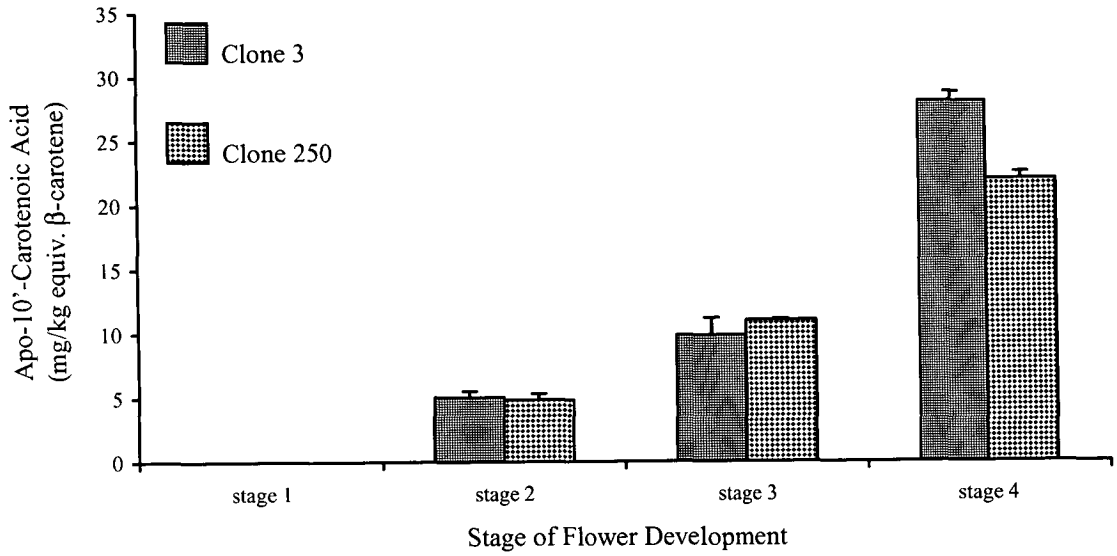


Figure 3.14 Changes in the levels of Apo-10'-Carotenoic Acid (expressed as β-carotene equivalent) in boronia clones 3 and 250.

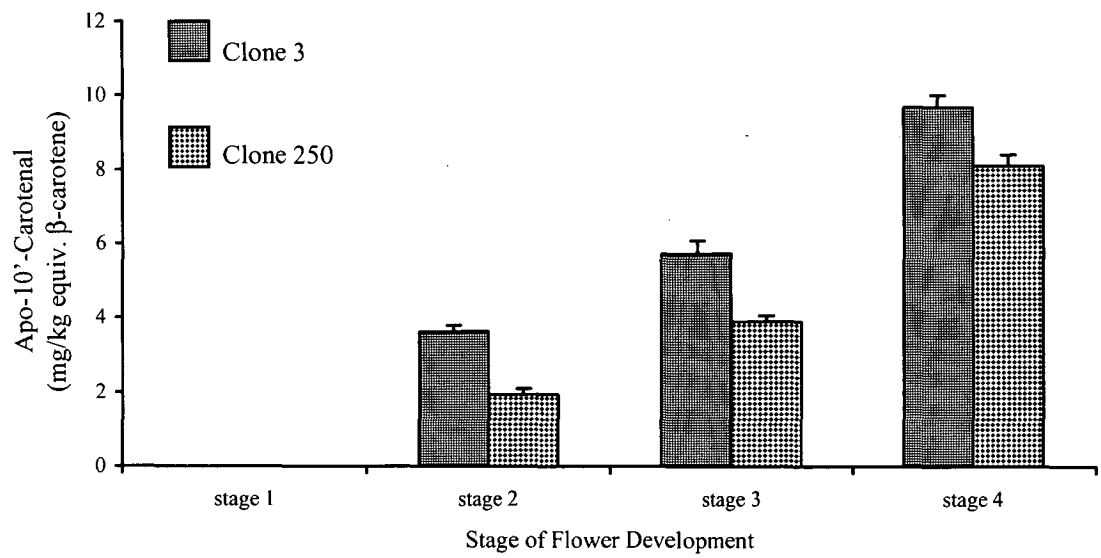


Figure 3.15 Changes in the levels of Apo-10'-Carotenal (expressed as β-carotene equivalent) in boronia clones 3 and 250.

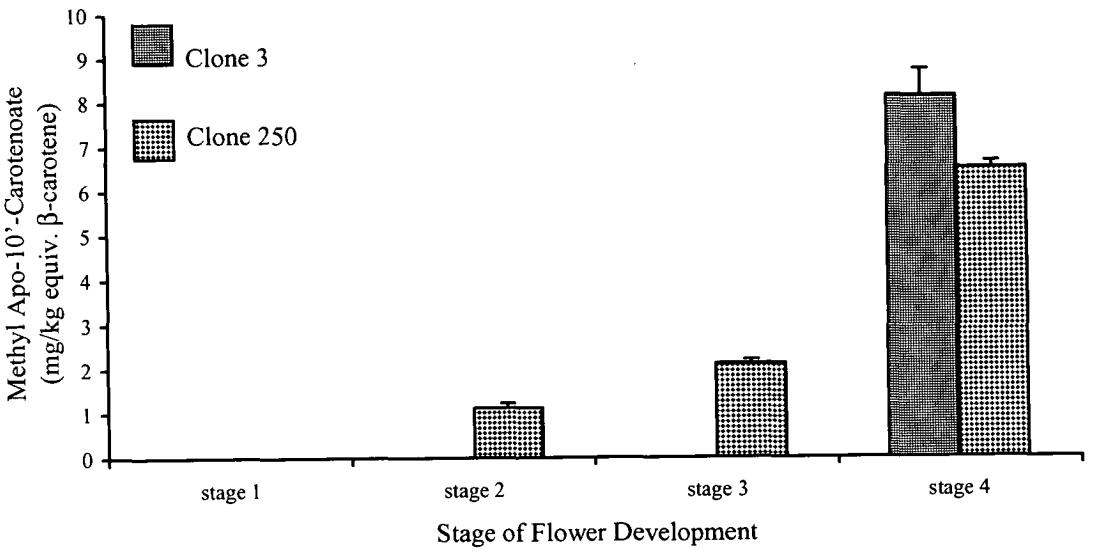


Figure 3.16 Changes in the levels of Methyl Apo-10'-Carotenoate (expressed as β-carotene equivalent) in boronia clones 3 and 250. No data was extracted for clone 3 at stages 2 and 3.

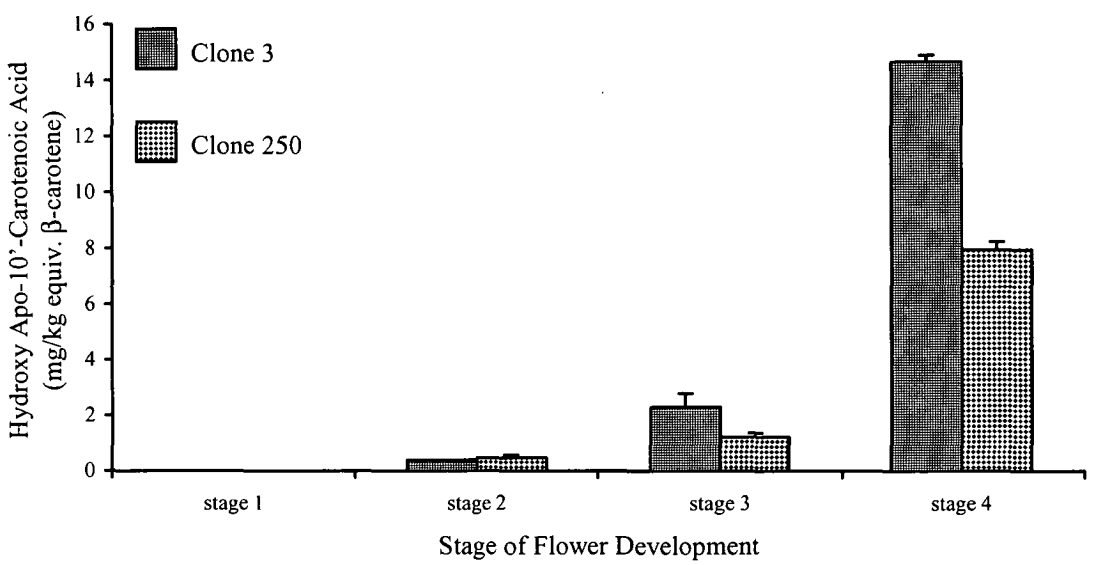


Figure 3.17 Changes in the levels of Hydroxy Apo-10'-Carotenoic Acid (expressed as β-carotene equivalent) in boronia clones 3 and 250.

In relation to xanthophyll cleavage it may be important to consider the activity of carotenoid cleavage enzymes in boronia to the relative biosynthesis of β-ionone and hydroxylated ionones. Certainly hydroxylated ionones are found at much lower levels in the extract than β-ionone. However, the reason for these lower levels may not be due to lower carotenoid cleavage activity against xanthophylls but rather be due to glycosylation of the hydroxylated compounds and their resultant unavailability as extract volatiles.

As noted in the literature review, there are no C-14 molecules such as rosafluene which would have supported the rationale that double cleavage of C-40 carotenoids occurs in boronia. However, the further metabolism of C-27 apo-carotenoids needs to be considered because the compounds do not appear to accumulate at a level commensurate with C-13 norisoprenoid biosynthesis. Changes in the levels of the hypothesised palmitic acid ester of hydroxy apo-10'- β-carotenoic acid (unknown carotenoid C) were also measured during flower development (**see figure 3.18**). This compound increased from 1.8 mg / kg in large buds to 4.6 mg / kg in open flowers. The pattern of increase for this compound was different to that for hydroxy apo-10'-β-carotenoic acid in that stronger increases relative to open flowers were apparent in the large bud and newly opening bud stages. However the onset of appearance of this compound correlated with the first appearance of the positively identified C-27 apo-

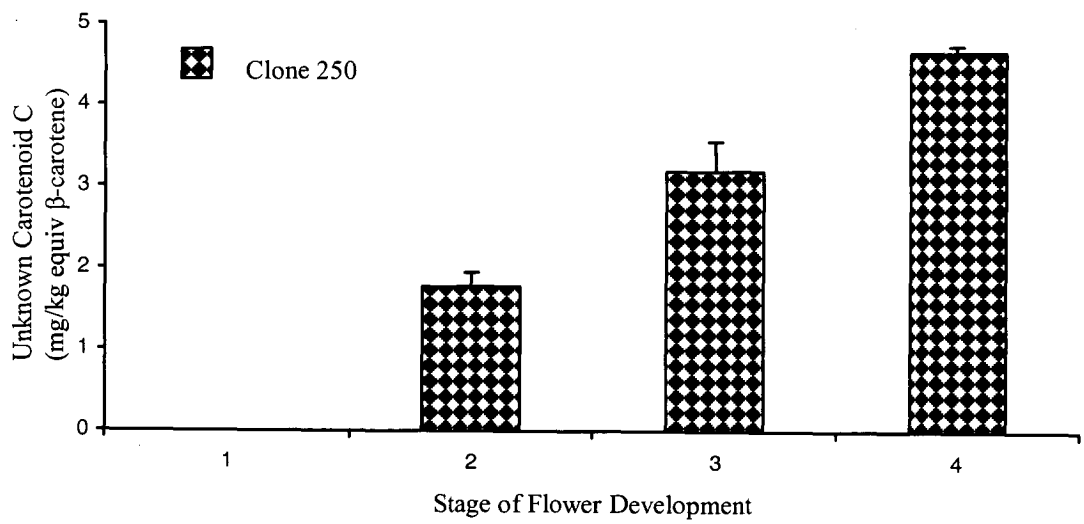


Figure 3.18 Changes in the levels of Unknown Carotenoids C (expressed as β-carotene equivalent) during flower development in boronia clone 250.

carotenoids. This has two implications. Firstly, it further supports the rationale that this compound is a C-27 apocarotenoid ester. Secondly if the tentative identification was accepted it would imply that further metabolism of apocarotenoids does occur in boronia flowers.

Overall, the results showed that the onset of metabolic activity in relation to carotenoid cleavage was initiated just prior to bud opening with large increases occurring between just opened buds and open flowers. The question of where aroma compounds are synthesised has been ongoingly debated in the literature. Winterhalter and Skouroumounis (1997) refer to a theory they attribute to Watanabe *et al.* (1993) suggesting that volatiles are synthesised in the leaves and transported to flowers as the glycoside. However this study supported the rationale that C-13 norisoprenoids are synthesised in the flowers. That evidence included the occurrence of C-27 apocarotenoid co-metabolites in flowers at the flower opening stage. This appearance is matched by increases in β-carotene and β-ionone at the same flower development stage. There appears to be a rapid turning on of biosynthesis during the initiation of flower opening. In addition, leaves analysed at the time of flowering did not show any evidence of carotenoids other than those C-40 carotenoids normally associated with chloroplasts.

Chapter 4

Screening for Glycosidic Conjugates in Boronia

4.1 Introduction

The previous chapters have detailed:
(1) The identification of C-27 apocarotenoids in boronia, which provided evidence that C-40 carotenoids were precursors of C-13 norisoprenioids. This evidence was based on the "complementary" nature of the C-13 and C-27 compounds with regard to proposed C40 carotenoid precursors.
(2) The potentially related changes in C-13 norisoprenoids, C-27 apocarotenoids and C-40 carotenoids occurring during flower development.

It was especially noted, that relative to known xanthophylls, hydroxy C-27 apocarotenoids structurally complemented known C-13 norisoprenoid alcohols that are found in boronia in low concentrations compared to β-ionone. In addition, there is abundant evidence in the literature that diverse C-13 norisoprenoids form glycosidic conjugates in many plant species (Winterhalter and Schreier, 1994; Williams *et al.*, 1992). Furthermore, preliminary experiments by MacTavish *et al.* (2002) had provided some evidence for the occurrence of these compounds in boronia flowers. This was based on the release of C-13 norisoprenoids from boronia extracts that had been incubated with protein extracts also isolated from boronia. C-13 norisoprenoids identified in boronia (Weyerstahl *et al.*, 1995) that have the potential to form glycosidic conjugates are β-ionol, 7,8-dihydro-β-ionol, 3-hydroxy-5,6-dihydro-β-ionone, 3-oxo-5,6-dihydro-βionol, 4-hydroxy-β-ionone and 3-hydroxy-β-ionone and 4-oxo-β-ionol. The structures of these compounds are detailed in **figure 1.4** of the literature review. The initial focus for this chapter then was the development of a method for screening boronia flowers for glycosides of C-13 norisoprenoids. The availability of tandem mass spectrometry as part of University of Tasmania facilities provided an opportunity to undertake this screening process.

4.2 Methods and Materials

4.2.1 Extraction of Boronia Marc

Initial Methanol Extraction

Boronia marc was initially extracted with methanol. Accordingly up to 100 g of boronia marc was blended with 250 mL of ice-cold methanol in a compressed-air driven steel blender for two minutes. The homogenate was further blended in batches with a Sorvall Omni-mixer at the highest speed for 1 minute using 4 x 25mL aliquots of methanol to transfer the suspension. This mixture was then shaken for 20 min at 200 cycles / s using an Ika-Werk (Janke and Kunkel) rotary shaker before filtering with a buchner filter (Whatman No. 1). The solids were washed with 4 x 25 mL (used for transfer) and 2 x 50 mL of methanol to yield 470 mL of extract. On some occasions a smaller amount of marc was extracted.

Further Clean-up

A further clean up procedure was developed involving re-extraction of the extract with distilled water. Accordingly the methanol was then removed by rotary evaporation which left a wet solid exhibiting a brown / purple colouration. The solids in the round bottom flask from the rotary evaporation step were shaken with 50 mL of distilled water until a slurry formed which was then filtered using a buchner funnel (Whatman No. 2, 2 layers). The remaining solids were washed with 2 x 50 mL aliquots of distilled water to yield 160 mL of a purple filtrate. This was stored overnight at 4°C prior to application on an XAD-2 column.

4.2.2 XAD-2 Chromatography

Column preparation: Amberlite XAD-2 (Supelco, 450 g) was suspended in distilled water and the fines removed with several washes. The resin was poured into a glass chromatography column (40 cm x 4.0 cm i.d.) that was fitted with a stopcock and upper reservoir. The resin was held in place using a glass wool plug and a bed of

acid-washed sand. The column was eluted with several column volumes of distilled water and then methanol before re-equilibration with distilled water prior to use.

Chromatography of the extract: The glycosidic extract (320 mL) was poured onto the prepared XAD-2 column and washed in with 3 x 20 mL of distilled water. The column was eluted with 4 L of distilled water to remove sugars. The glycosidic fraction was eluted with 4 L of methanol which was removed by rotary evaporation to yield typically 3.3 – 3.8 g of a purple solid. This glycosidic extract was stored at – 10°C prior to use. This method was developed from trials with smaller columns measuring 20 cm x 1.5 cm (i.d.). Correspondingly smaller volumes were used for the trial columns.

4.2.3 Sephadex G-10 Chromatography

A 20 cm x 1.5 cm glass column fitted with a sintered glass filter and a stopcock was used for the preparation of a Sephadex G-10 column. The resin (20.0 g) was equilibrated overnight in distilled water and washed several times to remove the fines. The resin, in the form of a slurry, was then poured into the column containing 5 cm of distilled water. After the resin had settled the column was washed with 500 mL of distilled water. Extracts from boronia were applied to the column and eluted with methanol. Fractions (10 mL) were analysed using HPLC / MS.

4.2.4 High Performance Liquid Chromatography

Extracts of boronia were chromatographically separated using a Waters 2690 HPLC. The HPLC was equipped with a Waters Novapak C18 3.9 mm x 150 mm column which was fitted with an Alltech Econosphere C18 guard cartridge. Three programs were used during the development of a suitable analytical method. Programs B and C became the routine HPLC methods.

Program A

The mobile phases were A. methanol (100%) and B. water. The initial conditions were 10% A / 90% B with a linear gradient to 90% A / 10% B at 50 minutes. The flow rate was 0.8mL / min.

Program B

The mobile phases were A. methanol (100%) and B. 0.1 M ammonium acetate (pH 6.38). The initial conditions were 10% A / 90% B with a linear gradient to 90% A / 10% B over 25 minutes. The flow rate was 0.8mL / min and injection volumes were usually 5 uL.

Program C

The mobile phases were A. methanol (100%) and B. 0.1 M ammonium acetate (pH 6.38). The initial conditions were 30% A / 70% B with a linear gradient to 70% A / 30% B at 40 minutes. The flow rate was 0.8 mL / min with injection volumes usually set at 5uL.

4.2.5 Mass Spectrometry

Mass spectral data was obtained with a Finnigan LCQ equipped with an atmospheric pressure chemical ionisation (APCI) ion source. Typical settings were: Vaporiser Temperature 450°C; Capillary Temperature 170°C; Sheath Gas Flow 60psi; Capillary Voltage 41V. Full scan analysis was usually over the range *m/z* 150 – 750 at 1 cycle / s and Finnigan Navigator software was used to generate and analyse the data. The software enabled data dependent selection of the most intense ion in a full scan spectrum followed by collisional activation to generate its product ions. The APCI technology used here allows this process to be repeated multiple times (MS^n). The trials discussed here utilised data dependent MS^2 and MS^3 experiments. Targeted experiments which allowed ions to be selected for MS^2 and MS^3 studies were also conducted. When single ions were targeted or selected in data dependent experiments an isolation window of at least 3 *m/z* units was used. In some experiments related ions of 2 *m/z* units difference were targeted with an isolation window of 6 *m/z* units.

4.3 Results and Discussion

4.3.1 HPLC / MS Analysis

Methanol extracts of boronia (without further purification) were initially analysed by full scan MS with alternating data dependent MS / MS. It was anticipated that the full scan data would show evidence of glycoside $[M + H]^+$ ions and that the MS / MS spectra of these ions would include the corresponding [aglycone + $H]^+$ ions. However, due to interference from large amounts of rutin in the methanol extract the data dependent scans did not show any evidence of glycosidic conjugates because the rutin and related flavanone peaks eluting nearby were automatically selected.

However the mass spectra in those runs indicated the presence of *m/z* 371 and *m/z* 373 peaks (equivalent to glycoside $[M+H]^+$ ions) together with ions that would be expected for the aglycone fragments represented by [aglycone $+H]^+$ at *m/z* 209 or 211 and corresponding to C-13 norisoprenoids of MW 208 or 210 respectively. Compounds previously identified in boronia with those molecular weights were 3-hydroxy-5,6-dihydro-β-ionone and 3-oxo-5,6-dihydro-β-ionol (MW = 210); and 4-hydroxyβ-ionone, 3-hydroxy-β-ionone, and 4-oxo-β-ionol (MW = 208).

Two strategies were then employed to overcome the problem with interference by the rutin compounds. The problem was examined through (1) changes to the separation procedure in order to reduce the amount of rutin in the extract and (2) through targeted MS^2 or MS^3 experiments on the possible parent ions in the methanol extract. These screening experiments were assisted through the observation that HPLC runs with methanol and water performed with residual ammonium ions (from previous HPLC experiments with ammonium acetate buffers) in the system resulted in the formation of ammonium adducts.

An overall summary of these experiments is shown in **figure 4.1** whereby it was demonstrated that the aglycone ionisation fragments could be obtained through MS^2 experiments from the $[M + H]^+$ or MS^3 experiments from the ammonium adduct

($[M+ NH_4]^+$). A more detailed examination of the screening process is then presented.

Mass Spectra

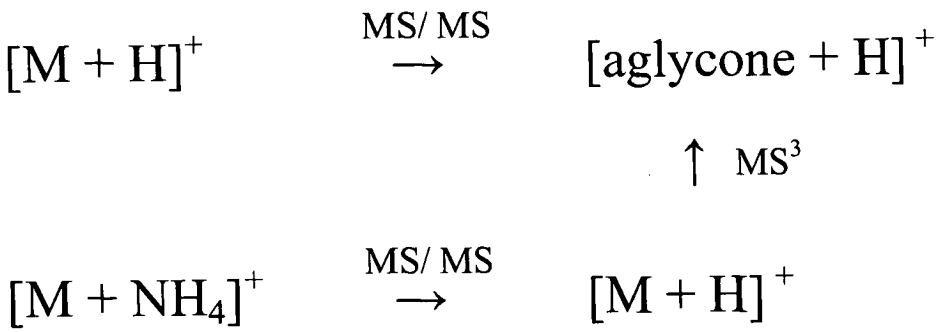


Figure 4.1 Summary of MS^n experiments showing ionisation of parent compounds and adducts to the aglycone ion.

Experiments were conducted, using HPLC method A, which screened for hexose glycosides containing aglycones with molecular weights of either 208 or 210. Accordingly, mass chromatograms were extracted from HPLC / MS experiments which specifically targeted $[aglycone + H]^+$ ions derived from $[M + H]^+$ ions in an MS^2 experiment or from $[M+ NH_4]^+$ via $[M + H]^+$ ions in an MS^3 experiment. These mass chromatograms are presented in **figure 4.2** and demonstrate alignment between several peaks resulting separately from the MS^2 and MS^3 experiments. Mass spectra were then obtained for the peaks labelled (a) through to (h) in **figure 4.2**. **Figure 4.3** shows the mass spectra for peaks a and e. Both spectra showed the most abundant ion at *m/z* 209 which corresponds to an aglycone with a molecular weight of 208. The hydroxylated ionones previously identified (Weyerstahl *et al.*, 1995) as present in boronia with this molecular weight are 3-hydroxy-β-ionone and 4-hydroxy-β-ionone. **Figure 4.4** shows the mass spectra obtained from peaks b and f in **figure 4.2**. These spectra have a high level of similarity to those obtained from peaks a and e. Both mass spectra obtained from the MS^2 experiment also showed an ion at *m/z* 191 for the loss of water from *m/z* 209.

Figure 4.5 shows mass spectra obtained from mass chromatogram peaks d and h in **figure 4.2**. Both spectra show ions at *m/z* 211 which corresponds to an aglycone of

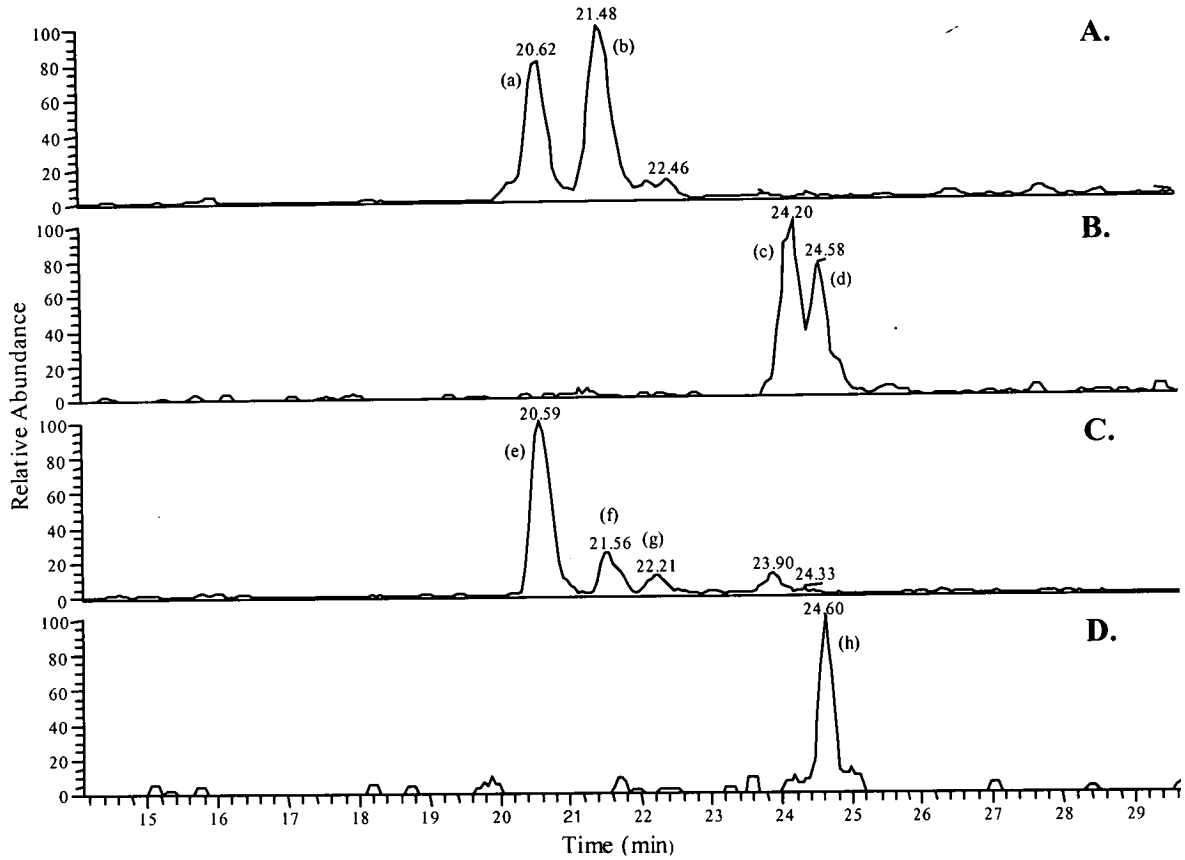


Figure 4.2 Mass chromatograms from specific MS^n experiments targeting the aglycone product ions of the C-13 norisoprenoid glycosides.

A. Mass chromatogram of *m/z* 209 ([aglycone + H]$^{+)}$ ions derived from $[M+H]^+$ at *m/z* 371.
B. Mass chromatogram of *m/z* 211 ([aglycone + H]$^+$) ions derived from $[M+H]^+$ at *m/z* 373.
C. Mass chromatogram of *m/z* 209 [aglycone + H]$^+$ ions derived in an MS^3 experiment from $[M+H]^+$ at *m/z* 371 in turn derived from the $[M+NH_4]^+$ at *m/z* 388.
D. Mass chromatogram of *m/z* 211 [aglycone + H]$^+$ ions derived in an MS^3 experiment from $[M+H]^+$ at *m/z* 373 in turn derived from the $[M+NH_4]^+$ at *m/z* 390.

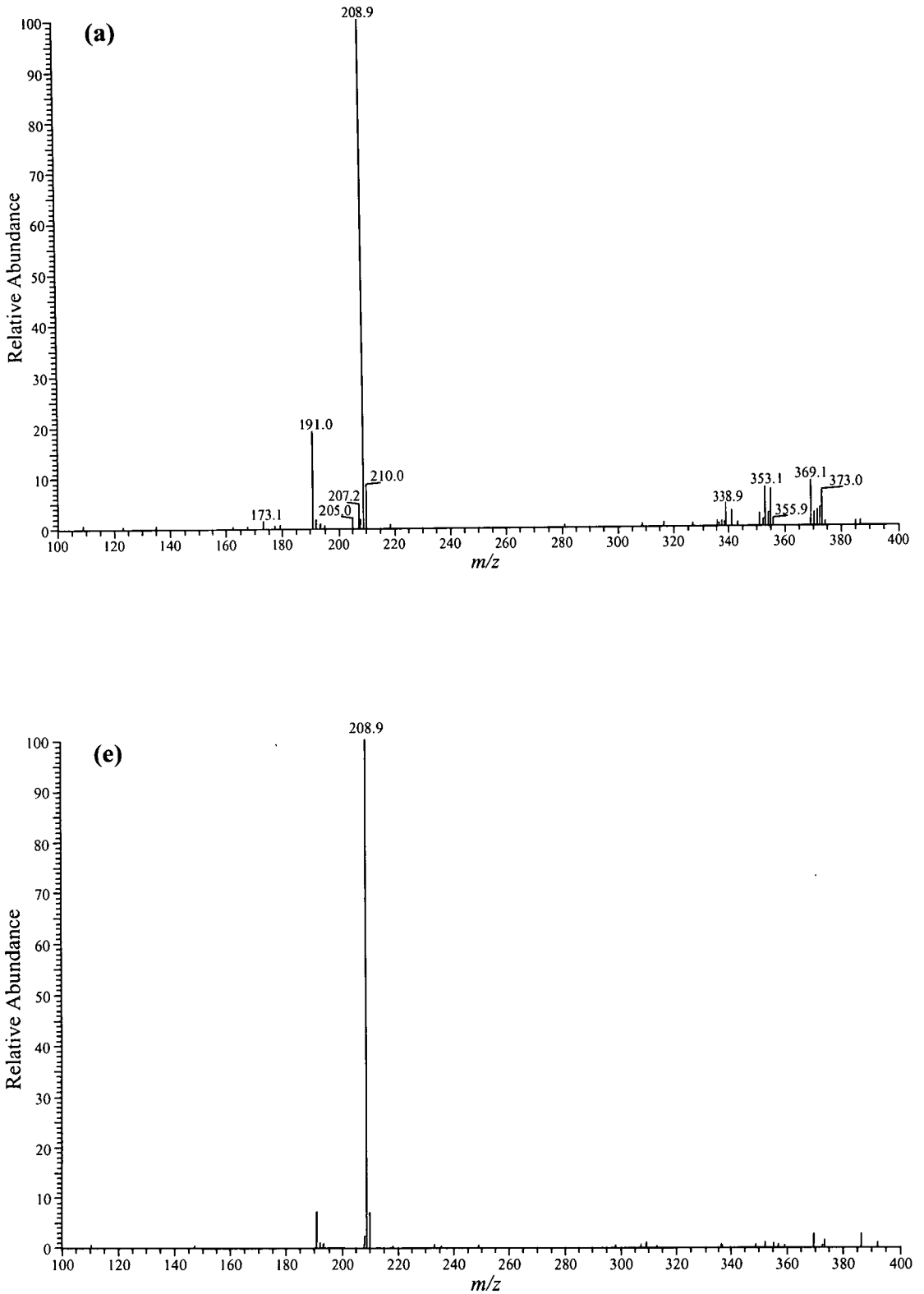


Figure 4.3 Mass spectra of peaks a and e from Figure 4.2

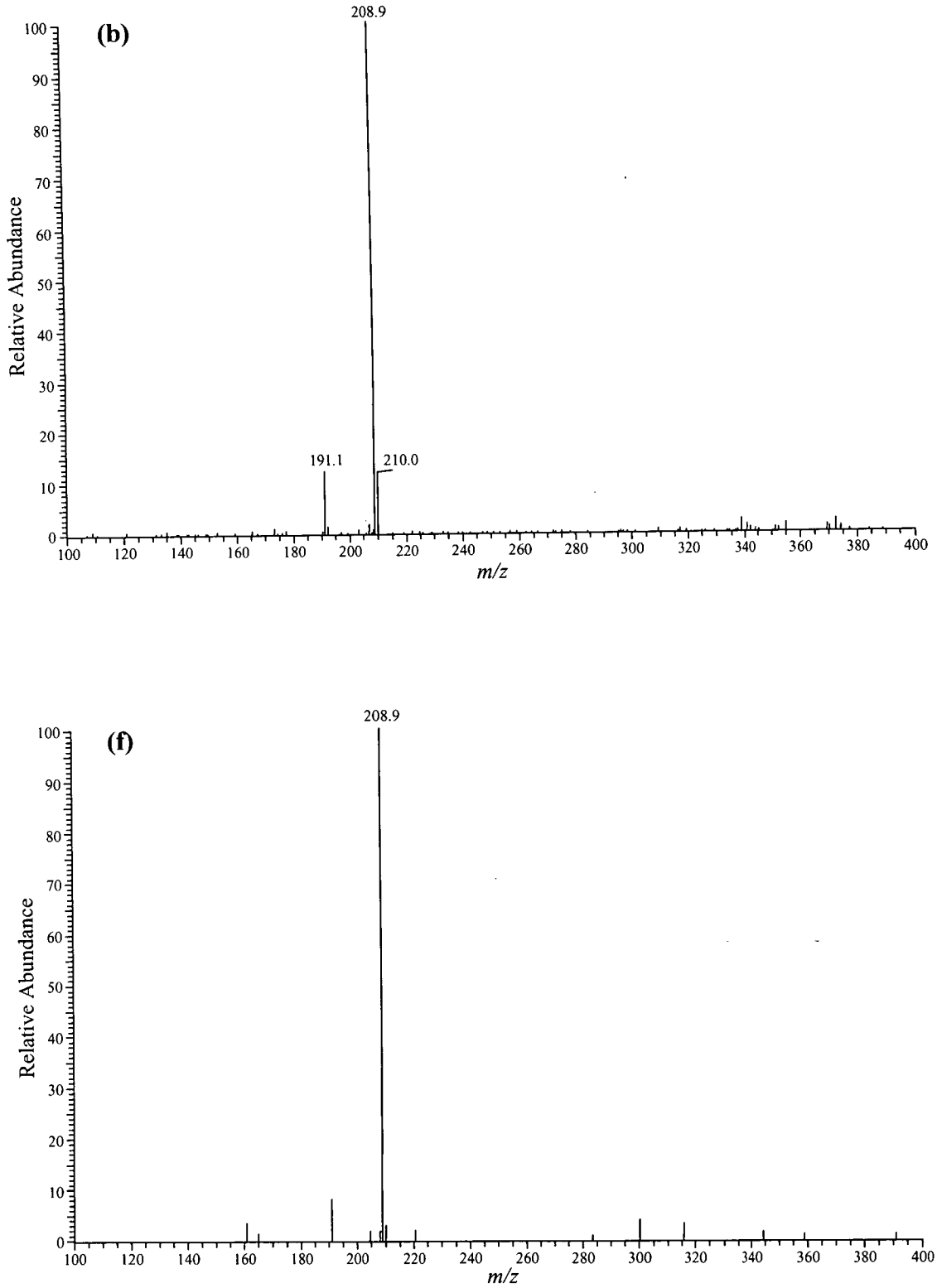


Figure 4.4 Mass spectra of peaks b and f from Figure 4.2

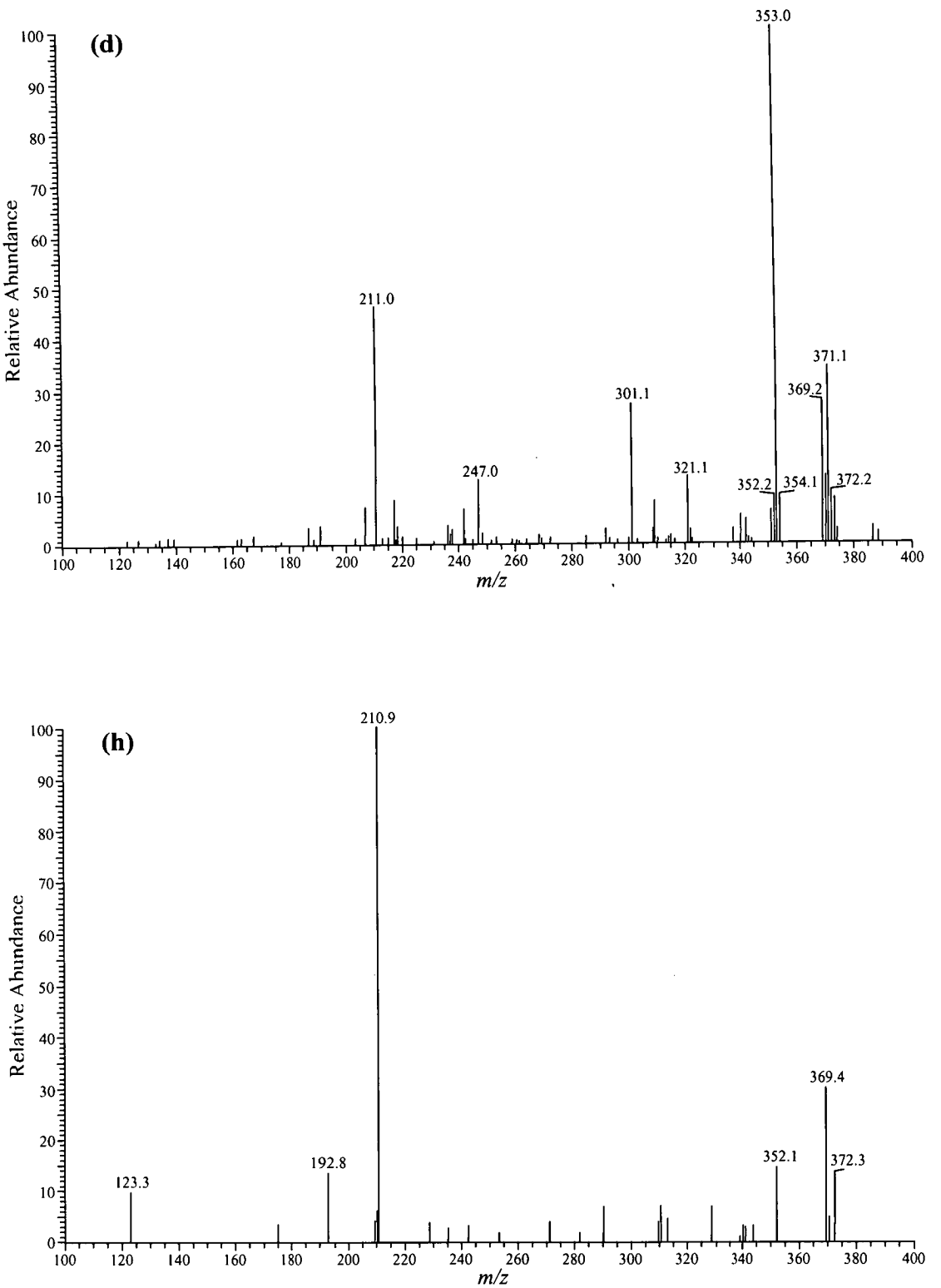


Figure 4.5 Mass spectra of peaks d and h from Figure 4.2

molecular weight 210. Both 3-hydroxy-β-ionone and 9-hydroxymegastigm-7-en-3-one are previously identified boronia volatiles (Weyerstahl *et al.*, 1995) that have this molecular weight. Importantly, this experiment confirmed that the same ions, equivalent to the various C-13 norisoprenoids, can be obtained from either the $[M + H]^+$ or ammonium adducts through MS^2 and MS^3 experiments respectively as outlined in **figure 4.1**.

The experimental data obtained during the initial experiments had additionally indicated that ions at *m/z* 209 and 211 equivalent in mass to C-13 norisoprenoid $[aglycone + H]^+$ ions were obtained from:

1. Ions of *m/z* 474 and 476 through MS^3 experiments and *m/z* 457 and 459 from MS^2 experiments.
2. Ions of *m/z* 492 through MS^3 experiments and *m/z* 475 from MS^2 experiments.

These mass differences were examined with regard to the molecular weights of alcohols previously identified in boronia. This allowed speculation that malonyl glycosides (Withopf *et al*, 1997) of C-13 norisoprenoids and cucurbates (MW = 474) were contributing to these outcomes. Cucurbates (MW = 226) were considered here through reasoning that the $[aglycone + H - H_2O]^+$ ion for the cucurbates was equivalent in mass to the $[aglycone + H]^+$ ion of C-13 norisoprenoids. Consequently an experiment using 0.1 M ammonium acetate as the more polar mobile phase (HPLC Method B), which specifically targeted the parent ions was designed to screen specifically for malonyl glycosides of C-13 norisoprenoids and cucurbates.

Figure 4.6 shows the total ion count (A.) and selected mass chromatograms (B.) for ions at *m/z* 209 and 211 (equivalent to $[aglycone + H]^+$) coming from the ammonium adducts of molecular ions (equivalent to $[M + NH_4]^+$) corresponding to the putative malonyl glycosides of C-13 norisoprenoids and (C.) ions of *m/z* 209 (equivalent to $[aglycone + H - H_2O]^+$) and 227 (equivalent to $[aglycone + H]^+$) coming from the ammonium adduct of the molecular ion corresponding to malonyl glycosides of cucurbates. Typical mass spectra, which further supported the assignment of these compounds as malonyl glycosides of C-13 norisoprenoids and cucurbates, are presented in **figure 4.7**.

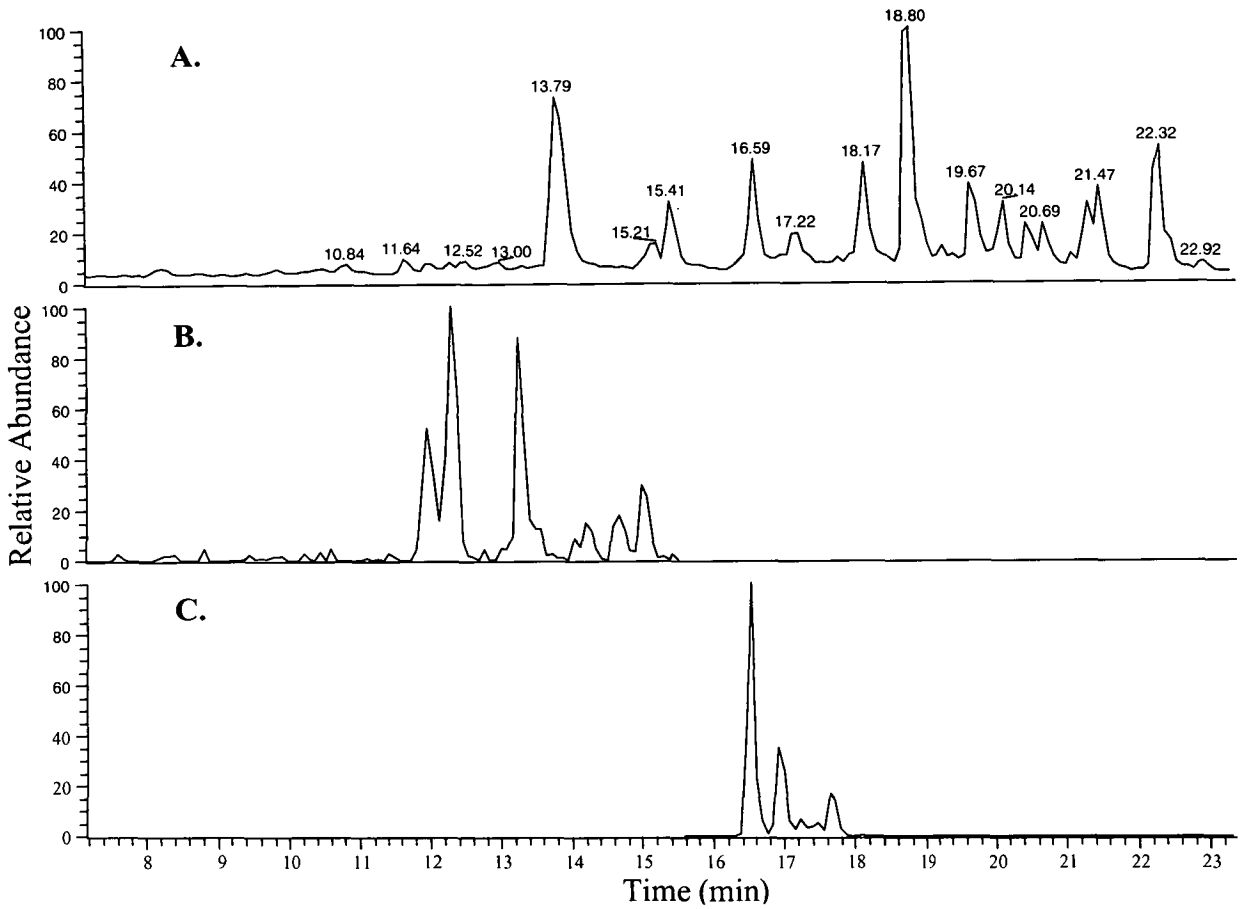


Figure 4.6 Chromatographic results of malonyl glycoside screen. A. TIC B. Selected MS/ MS mass chromatograms (209, 211coming from 474, 476) and C. Selected MS/ MS mass chromatograms (209, 227 coming from 492)

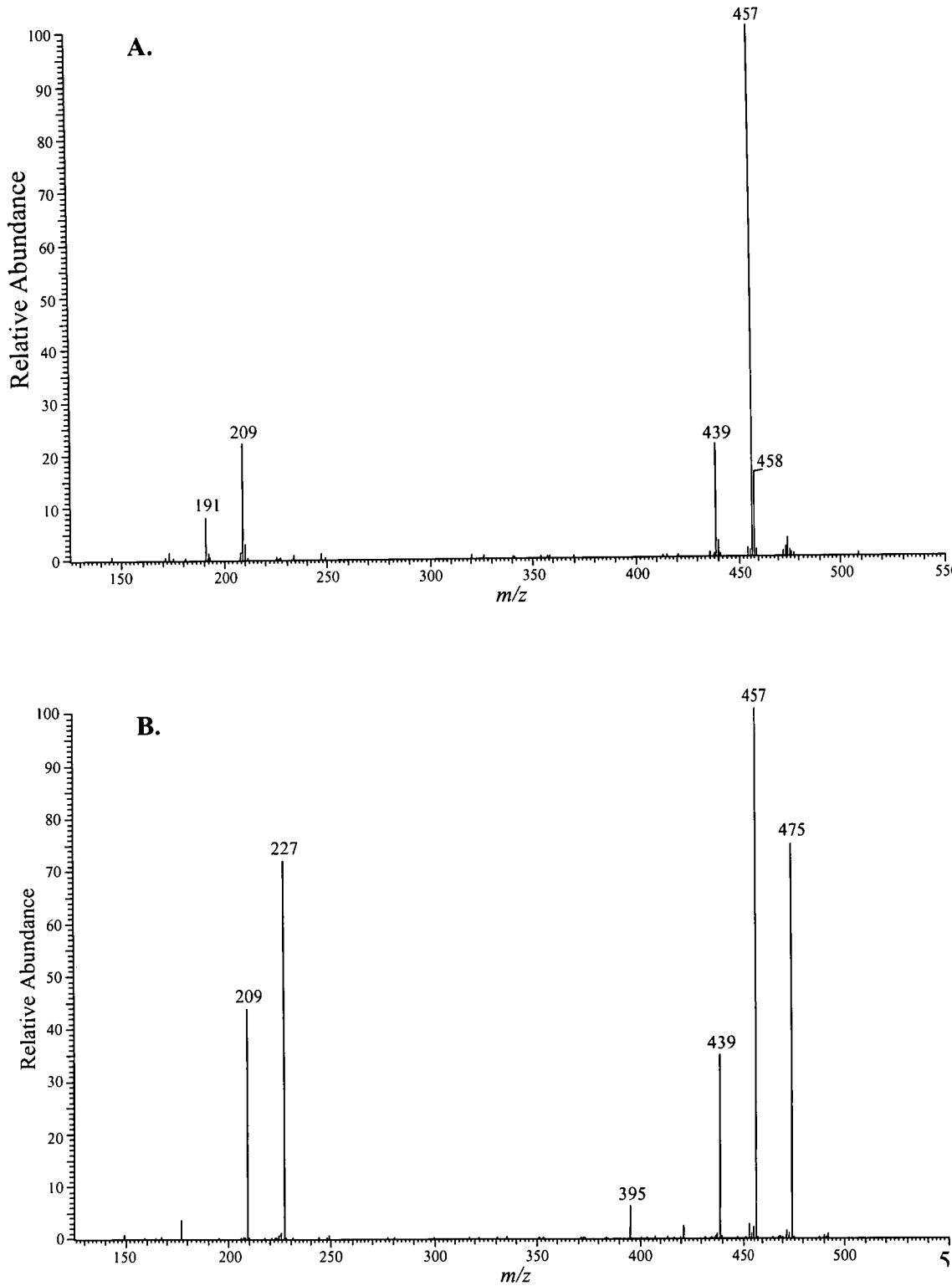


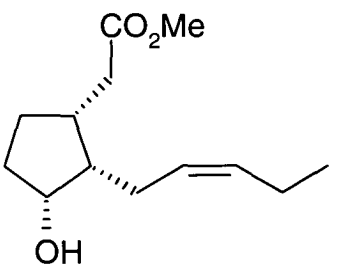
Figure 4.7 Mass spectra showing typical MS / MS patterns for putative malonyl glycosides of A. C-13 norisoprenoids, and B. cucurbates.

The MS / MS spectrum for the proposed malonyl glycosides of C-13 norisoprenoids (**figure 4.7, A**) showed peaks for the loss of ammonia at *m/z* 457 and for a further loss of water at *m/z* 439. Further peaks at *m/z* 209 and 191 represented the [aglycone + H]$^+$ and [aglycone + H – H_2O]$^+$ ions respectively. A typical MS / MS spectrum for the proposed malonyl glycosides of cucurbates (**figure 4.7, B**) showed a similar level of detail with peaks for the loss of ammonia at *m/z* 475, a further loss of water at *m/z* 457, and the loss of two water molecules at *m/z* 439. The peaks at *m/z* 227 and 209 were consistent with the [aglycone + H]$^+$ and [aglycone + H – H_2O]$^+$ ions respectively which confirmed the earlier rationale in relation to considering the molecular weight evidence for cucurbates in boronia.

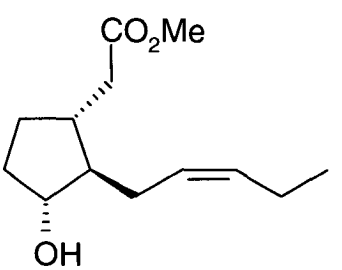
The first evidence in the literature for the presence of malonyl glycosides of compounds with flavour and aroma value was found as an aryl glycoside in papaya fruit (Schwab and Schreier, 1988). Moon *et al.* (1994) used NMR spectroscopy to demonstrate the presence of malonyl glycosides of linalool in the flower buds of jasmine. The more extensive and specific investigation of Withopf *et al.* (1997) found benzyl, 2-phenylethyl, geranyl, citronellyl, and 2,5dimethyl-4-hydroxy-3(2H)-furanone malonyl glycosides variously in the fruit, leaves and peel of several species. The authors confirmed the identities of these compounds using synthesised standards, and suggested that malonyl glycosides were ubiquitous in the plant kingdom, but that previously they had been overlooked as flavour precursors due to instability during sample preparation. However no previous evidence has been presented in the literature for malonyl glycosides of either C-13 norisoprenoids or cucurbates.

Whilst the results obtained in this study confirm the use of HPLC / MS as a screening procedure for malonyl glycosides previously similarly developed by Withopf *et al.* (1997) some methodological differences need to be noted. Firstly, the present study used atmospheric pressure chemical ionisation (APCI) as the ion source rather than electrospray ionisation (ESI) used by Withopf and colleagues. Secondly, Withopf *et al.* (1997) used synthesised standards to confirm the identities of the malonyl glycosides. Therefore in this study it is only possible to claim tentative assignment of the proposed conjugates. Further investigation will need to be undertaken to confirm the proposed identities.

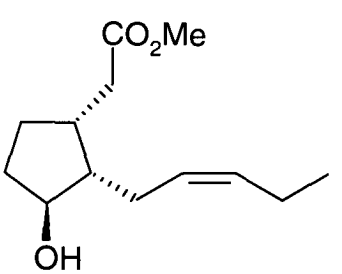
Whilst the structures of possible C-13 norisoprenoids were previously detailed in the literature review (**Figure 1.4**) the cucurbate compounds found in boronia are methyl esters of cucurbic acid and their structures are detailed in **Figure 4.8**. These compounds are alcohol analogues of the methyl jasmonates which are important in boronia because of their high value as flavour and aroma compounds.



Methyl 2,3-di-*epi*-cucurbate



Methyl 3-*epi*-cucurbate



Methyl cucurbate

Figure 4.8 The structure of cucurbates identified as occurring on boronia

Following the tentative assignment of glycosides of C-13 norisoprenoids and malonyl glycosides of C-13 norisoprenoids and cucurbates, the data from previous experiments was examined for evidence of conjugates of other alcohols found in boronia. An experiment designed to test the usefulness of Sephadex G-10 chromatography (see later discussion) as a step in the purification of the proposed glycosides provided evidence of both monosaccharide and malonyl glycosides of monoterpenes alcohols. Partial purification with Sephadex G-10 removed the interference of rutin and other polyphenolic compounds enabling a more detailed mass spectral analysis of the proposed conjugates. The HPLC / MS experiment (HPLC Method C) detailed here accumulated "data dependent" MS^n data from a glycoside rich fraction. **Figure 4.9** shows the mass chromatograms for selected ions corresponding to glycosides and malonyl glycosides of monoterpenols and monoterpenediols. Relevant mass spectra and MS / MS spectra were then obtained to confirm the identities of the various monoterpene glycosides.

A single peak was observed in the mass chromatogram for compounds corresponding to malonyl glycosides of monoterpenols (**figure 4.10 A**). The mass spectra obtained at this point in the TIC supported the assignation with a dominant ion peak corresponding to $[M + NH_4]^+$ at *m/z* 420. The chromatographic position of the proposed compound is in the same region as for the elution of malonyl glycosides of cucurbates and a smaller peak at *m/z* 492 was noted. The MS / MS spectrum (**figure 4.10 B**) which targeted the ion at *m/z* 420 included a peak at *m/z* 137 corresponding to $[\text{aglycone} + H - H_2O]^+$.

Three peaks were observed in the mass chromatograms for compounds corresponding to the malonyl glycosides of monoterpenediols. All three peaks gave mass spectra with the dominant ion at *m/z* 436. The mass spectrum and MS / MS spectra for the peak at 10.97 min (**figure 4.9 C**) are presented in **figure 4.11**. The MS / MS spectrum (**figure 4.11 B**) which targeted the predominant ion at *m/z* 436 showed the loss of an ammonium adduct to $[M + H]^+$ at *m/z* 419 and the further loss of water at *m/z* 401. In addition the peaks at *m/z* 153 ($[\text{aglycone} + H - H_2O]^+$) and at *m/z* 135 $[\text{aglycone} + H - 2 \times H_2O]^+$ were consistent with the proposed identity.

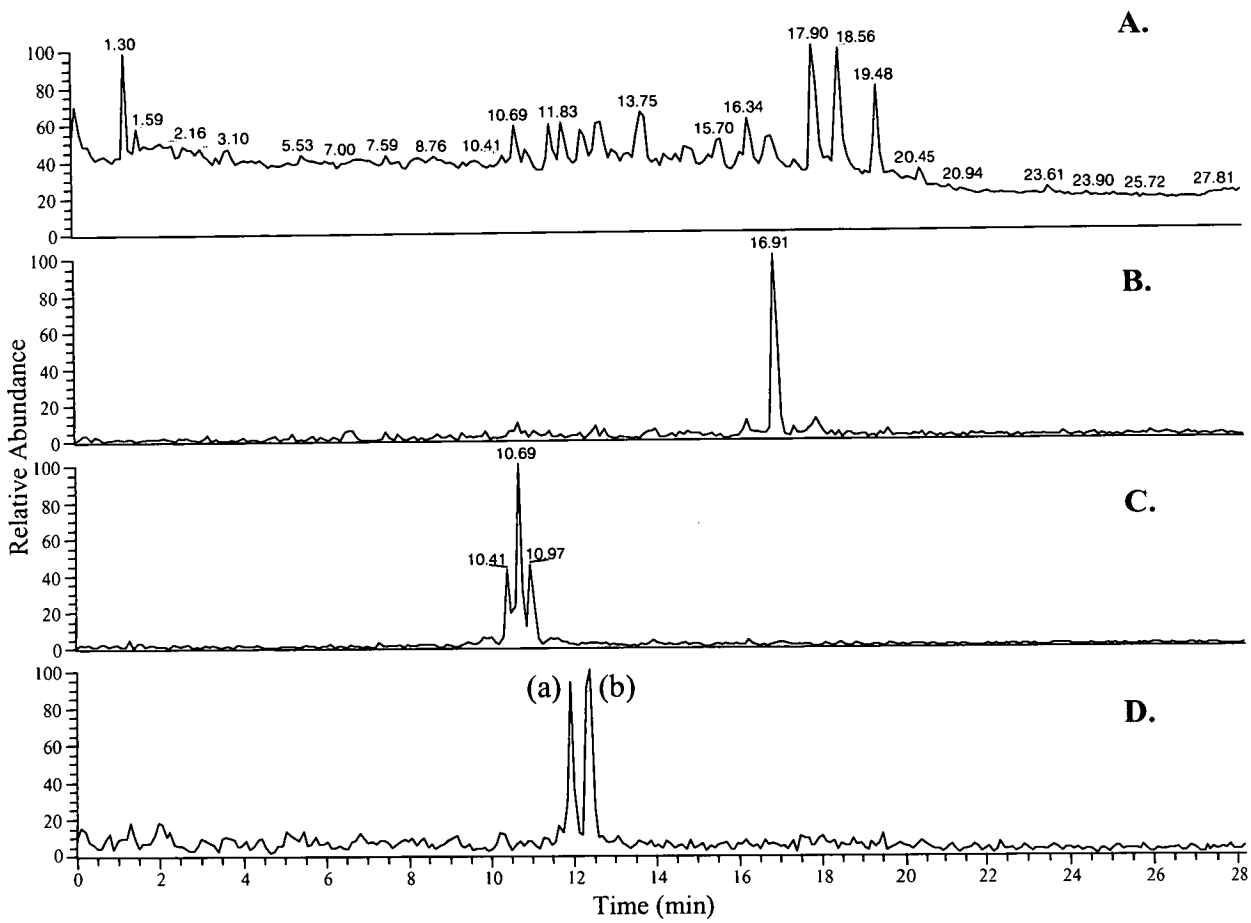


Figure 4.9 Chromatographic results of screening for monoterpenes.

E. TIC
F. Mass chromatogram of *m/z* 420, $[M + NH_4]^+$ ions corresponding to malonyl glycosides of monoterpenols.
G. Mass chromatogram of *m/z* 434 and 436, $[M + H]^+$ ions corresponding to malonyl glycosides of monoterpenediols.
H. Mass chromatogram of *m/z* 348 and 350, $[M + H]^+$ ions corresponding to glycosides of monoterpendiols

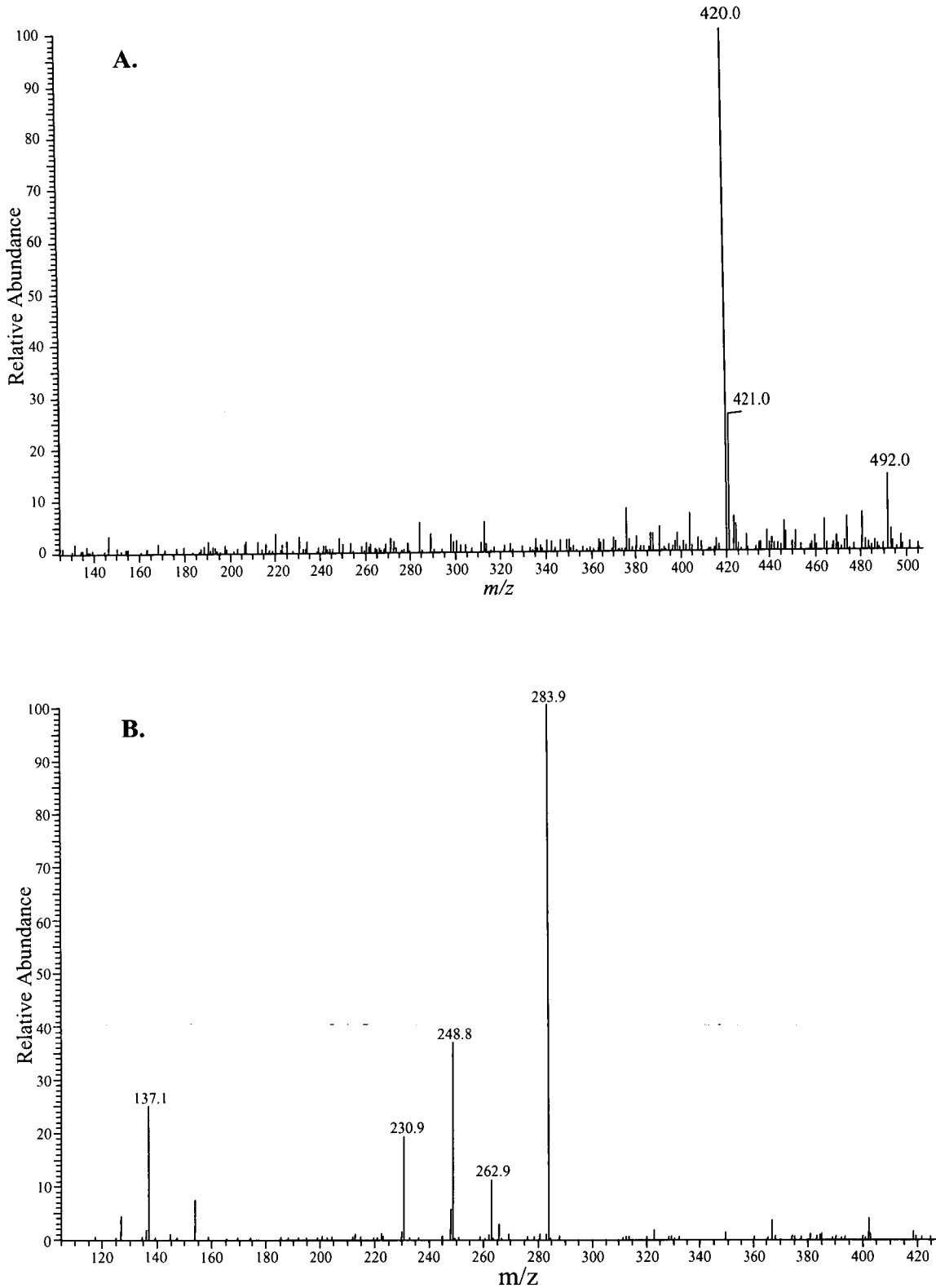


Figure 4.10 Mass spectrum (A) and MS / MS spectrum (B) derived from the peak (RT = 16.91) in figure 4.9 A for ions at m/z 420.

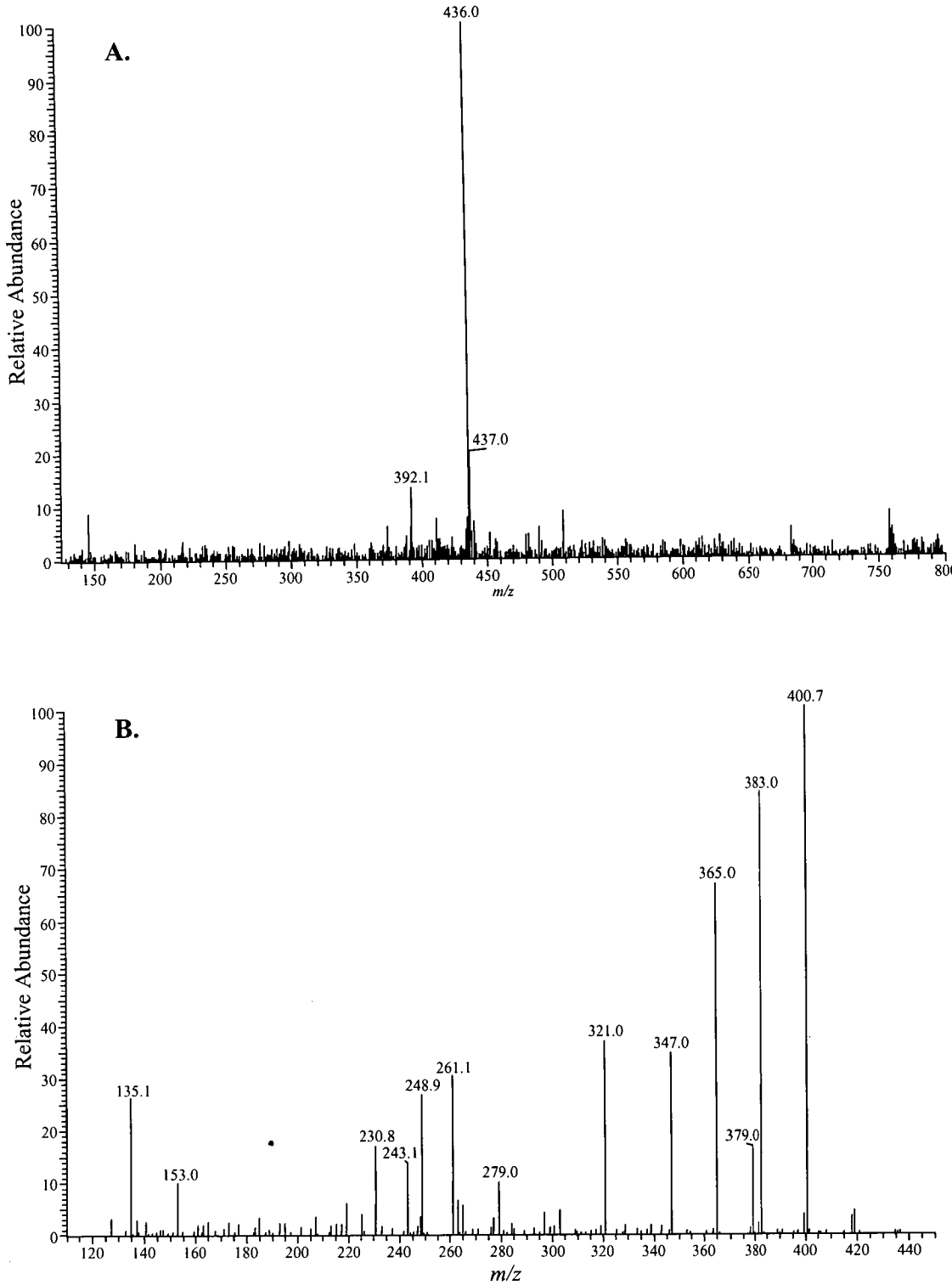


Figure 4.11 Mass spectrum (A) and MS / MS spectrum (B) derived from the third peak (RT = 10.97) in figure 4.9 C for ions at *m/z* 434 / 436.

Evidence for simple glycosides (containing one unmodified hexyl sugar group) of monoterpenediols was also obtained in this experiment. **Figure 4.9 D** is the mass chromatogram of ions at *m/z* 348 and 350. The mass spectra for these peaks are presented in **figure 4.12**. The peaks at *m/z* 350 are evidence of the monoterpenediol glycosides. However, MS / MS spectra were not obtained as this experiment was run in data dependent mode with the nearby elution of malonyl glycosides of C-13 norisoprenoids which gave stronger ions at *m/z* 474 and 476. It is important to note that the individual mass chromatograms for the peaks at *m/z* 474 and 476 showed they were not aligned with the peaks at *m/z* 350.

4.3.2 Further Purification of Glycosides

Flavanoid glycosides are widely distributed in the plant kingdom including in citrus fruit (Rouseff *et al.*, 1987) and Bazemore *et al.* (2003) speculated that separation of glycosides using XAD resins was made difficult in the presence of high concentrations of flavanone glycosides. The initial separative issue with regard to the screening of extracts for glycosidic conjugates in boronia was the presence of high levels of rutin and other polyphenolic compounds because as noted earlier, rutin interfered with the data dependent mass spectrometric experiments. A number of separative and chromatographic processes were investigated with regard to the further purification of boronia glycosides. The most successful techniques were re-extraction of the methanol extract with distilled water and Sephadex G-10 chromatography.

Re-extraction in distilled water of the methanol extract strongly increased the ratio of glycosides to rutin in the extracts. However a comparison of the TIC profiles in **figures 4.6 and 4.8** demonstrates the exceptional increase in purity provided by Sephadex G-10 chromatography as part of a process that might lead to the purification of boronia glycosides. The use of Sephadex G-10 chromatography was based on a paper by Nordström (1967) who found that polyphenolic glycosides, including rutin, were strongly absorbed onto this resin. By comparison an alternative process of using polyvinylpolypyrrolidone (PVPP) (Doner *et al.*, 1993) proved equivocal when tested as a separation method for the removal of polyphenols from

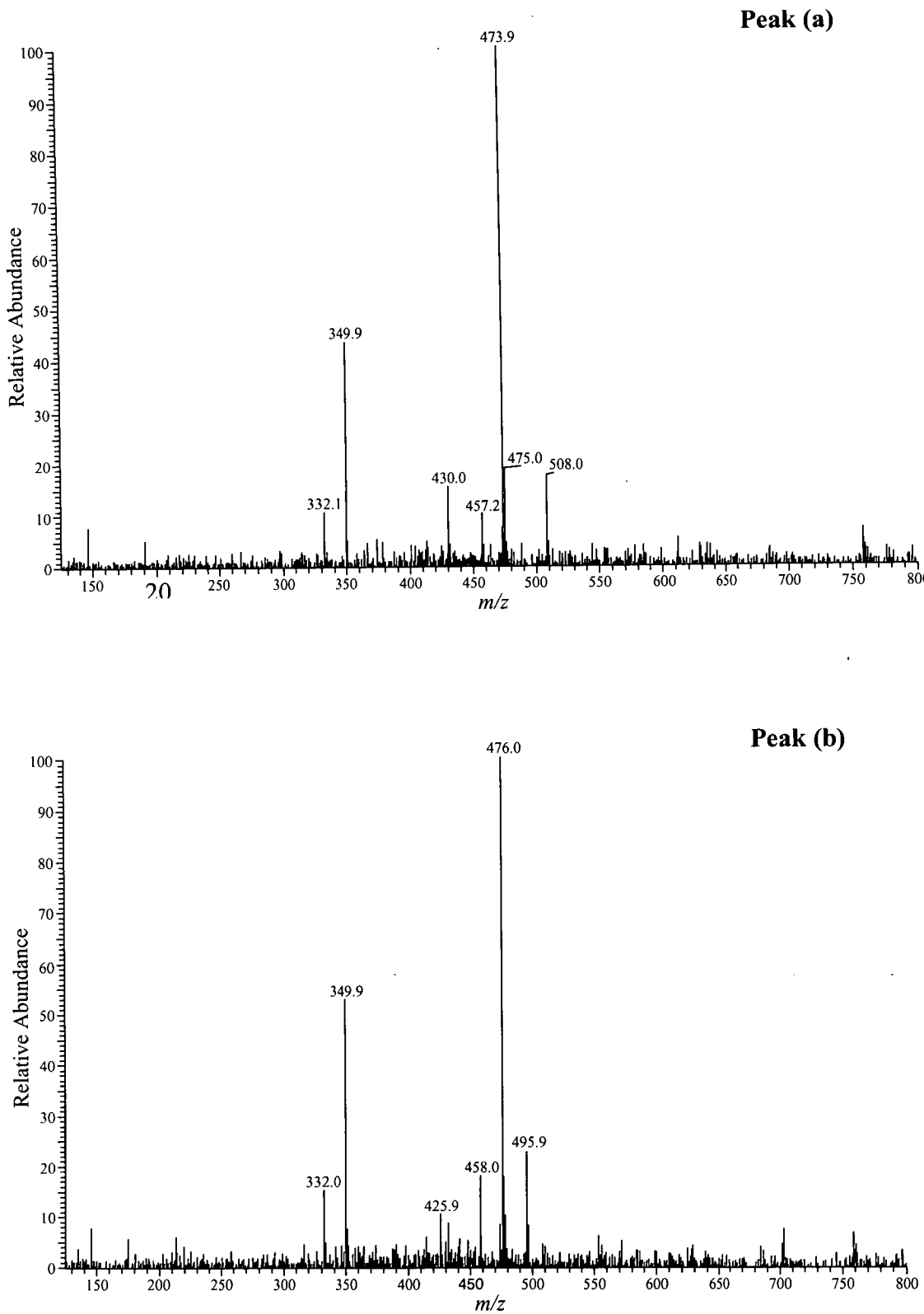


Figure 4.12 Mass spectra of the two peaks (a and b) representing glycosides of monoterpenediols.

boronia. Essentially rutin and other compounds eluting in the polyphenolic chromatographic region were not substantially removed following treatment with PVPP. Additionally there was evidence that malonyl glycosides were partly removed from solution by the PVPP treatment. This study was very preliminary and needs further work to gain a more comprehensive view. Other resins that have been used for the removal of poly phenols include Amberlite IRA900CI (D'alvise *et al.*, 2000).

A Sephadex G-10 chromatographic step would certainly prove useful as a step leading to the isolation of individual glycosides. Other chromatographic systems trialled on crude extracts included solvent extraction, silica chromatography, NH2 chromatography and semi - preparative HPLC on C18 columns. These further techniques gave variable results. It is likely that preparative HPLC on a reversed phase C18 medium would lead to the isolation of pure compounds.

4.4 Summary

In summary a method was developed that allowed scanning for six different types of glycosides tentatively identified in boronia extracts. This was achieved through selection of mass chromatograms for the ammonium adducts of each conjugate from the full scan data. The assignment of identities was supported through both the data dependent and targeted MS^2 and MS^3 experiments described here. Each of the glycosidic conjugates proposed is listed in **table 4.1** along with the product ions from the various MS experiments. The six glycosidic conjugates tentatively identified in this chapter included simple glycosides and malonyl glycosides. Initial progress was also made with regard to the further purification of boronia glycosides with chromatography on Sephadex G10 identified as significant step in the removal of polyphenols from the initial extracts.

Proposed glycosidic conjugate	$[M + NH_4]^+$	$[M + H]^+$	$[M+ H - H_2O]^+$	$[M+ H - 2 \times H_2O]^+$	$[Aglycone + H]^+$	$[Aglycone + H - H_2O]^+$	MW of conjugate	MW of aglycone
Malonyl glycoside of	474	457	439	421	209	191	456	208
C-13 norisoprenoids	476	459	441	423	211	193	458	210
Glycosides of C-13	388	371	353	335	209	191	370	208
Norisoprenoids	390	373	355	337	211	193	372	210
Malonyl glycosides of cucurbates	492	475	457	439	227	209	474	226
Glycosides of monoterpendiols	350	333	315	296	171	153	332	170
Malonyl glycosides of monoterpendiols	436	419	401	383	171	153	418	170
Malonyl glysosides of monoterpenols	420	403	385	367	155	137	402	154

Table 4.1 Summary of mass spectral ions found in MS^2 and MS^3 experiments which were part of the screening process for glycosidic conjugates in boronia.

Chapter 5

Enzymatic Hydrolysis of Glycosides

5.1 Introduction

The experimental data presented in the previous chapters indicated several important points that might guide the development of protocols with potential to lead to the release of aroma and flavour compounds from boronia marc. In summary the data discussed so far has shown that:

1. There is clear evidence through the appearance of hydroxylated C-27 apocarotenoids (Cooper *et al.*, 2003), that xanthophylls are subject to cleavage in the 9,10 position, which would result in hydroxylated C-13 norisoprenoid products that have the further potential to form glycosides.
2. There is a rapid turning on of carotenoid metabolism during flower development that is linked in time to the appearance of β-ionone.
3. Screening of boronia marc using HPLC / MS provides strong evidence for the presence of monoterpenol, cucurbate, and C-13 norisoprenoid glycosidic conjugates.

The combination of this new knowledge with an understanding from the relevant literature, that there are a wide range of hydroxylated compounds in boronia extract and that these compounds in other plant species are often found in high concentration in the bound or glycosidic form, provides a strong rationale for an exploration of the release of flavour and aroma compounds from the putative glycosidic conjugates proposed in boronia. With regard to this, enzyme hydrolysis studies have been used to confirm the presence of glycosidic conjugates in other plants. This chapter then, explores the release of conjugates through enzymatic hydrolysis using β-D-glucosidase and a commercially available pectinase (AR 2000) following the development of suitable methodology. It is intended that these experiments provide further clarification with regard to the identity of the proposed glycosidic conjugates.

5.2 Materials and Methods

5.2.1 Enzyme Hydrolysis Experiment

The glycosidic extract obtained from clone 3 boronia marc, using solvent extraction and XAD-2 chromatography as outlined in the methodology described in chapter 4, was tested in a model system against pectinase (AR2000) and β-D-glucosidase activity similarly to the method of Schneider *et al.*, (2001). Enzyme solutions, 10 mg of AR 2000 or 2 mg of β-D-glucosidase, in 1 mL of citrate phosphate buffer (pH 5.0) were added to a solution containing 10 mg of the extract such that the final incubation volume was 5 mL. The mixtures with six replicates were then incubated for 16 h at 40°C.

The experimental design used here was based on literature models (AR 2000: Schneider *et al.*, 2001; β-D-glucosidase: Groyne *et al.*, 1999). Preliminary experiments to determine appropriate enzyme and substrate quantities were conducted. Accordingly, enzyme activities for both β-D-glucosidase and AR 2000 (10mg of enzyme) were trialled against different amounts of octyl-β-D-glycoside (1 and 2 mg) and the XAD-2 extract (10 and 20mg). A further trial testing 1, 2 and 5 mg amounts of β-D-glucosidase against 10 mg of XAD-2 extract was conducted to optimise the β-D-glucosidase quantities.

5.2.2 Solvent Extraction

Previously with regard to boronia research, volatiles were extracted using petroleum ether (pentane / hexane; 80:20). However two factors implied the potential value in assessing the best solvent for extraction of volatiles obtained through enzymatic hydrolysis of the hydrolysate. They were:

1. Many of the volatiles obtained through the hydrolysis experiments were poly-oxygenated C-13 norisoprenoids and monoterpenes. These compounds

are likely to be more polar than the mono-oxygenated compounds usually assessed in boronia research.

2. An experimental methodology with regard to Solid Phase Micro Extraction (SPME) had been assessed for use in this study. The assessment indicated that unlike β-ionone the poly-oxygenated C-13 norisoprenoids did not bind well to either polydimethylsiloxane (PDMS) or carboxen SPME fibres thus implying the need for caution with regard to the existing analytical methodology used in boronia analysis.

The extraction efficiency of four solvents (Hexane / DCM 2:1; Hexane / DCM 1:2; DCM; Ethyl Acetate) was determined using an AR 2000 hydrolysate. Samples were extracted three times with 1.5 mL of solvent prior to analysis using GC / MS. The samples were kept at 4°C during the extraction process. The aqueous and solvent layers were clarified by centrifugation for 2 min at 4000 rpm using an MSE Super Minor centrifuge. Samples were extracted and centrifuged in 7 mL glass vials fitted with a silicon seal. Ethyl acetate was found to be the most suitable solvent with 99% extraction of non-polar and poly-oxygenated volatiles after three extractions. In addition the non-polar extraction solvents were found to be unsuitable for extraction of the poly-oxygenated compounds. The data did not show any solvent effect on the stability of the volatile components and ethyl acetate has been used previously as an extraction solvent for the extraction of C-13 norisoprenoids from an enzyme hydrolysate (Roberts *et al.*, 1994).

5.2.3 Data Analysis

High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS) Analysis

Aqueous samples containing the glycosidically bound volatiles were analysed using a Waters 2690 HPLC and Finnigan LCQ. The HPLC was equipped with a Waters Novapak C18 3.9 mm x 150 mm column. The mobile phases were: A. Methanol (100%) and B. 0.1M Ammonium Acetate. The initial conditions were 30% A / 70% B with a gradient to 90% A / 10% B at 25 min. Typical MS conditions were: APCI

source; Vaporiser: 470°C; Capillary: 175°C; Sheath Gas Flow: 60 psi; Capillary Voltage: 46 V. Scanning was from *m/z* 150 – *m/z* 750. MS^2 and MS^3 experiments as described in chapter 4 were conducted on relevant ions for further structural information when clarification was necessary.

Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

Ethyl Acetate extracts containing released volatiles were analysed with a Varian CP-3800 GC equipped with a VF-5ms 30 m x 0.25 mm i.d. x 0.25 μm film thickness Varian 1200 FactorFour® Capillary Column. Injections were made with a Varian 1177 split/splitless injector at a temperature of 210°C in splitless mode. The carrier gas was helium at a flow rate of 1.0 mL / min. The initial oven temperature was 40°C, held for 2 min, rising to 230°C at 5°C / min. Sample detection was performed with a Varian Triple Quadrupole Mass Spectrometer scanning from *m/z* 35 to *m/z* 350 at 4 scans per second in electron ionisation mode. Analysis was performed using a Varian Mass Spectrometry Workstation Version 6.30. Peaks were identified by a combination of commercial (NIST, version 2.0), in-house databases, and relevant literature in conjunction with Kovats' retention indices.

Gas Chromatography – FID Analysis

The data was analysed using a Hewlett Packard 5890 Series II GC equipped with a Flame Ionisation Detector (FID) and a VF-5ms 30 m x 0.25 mm i.d. x 0.25 μm film thickness Varian FactorFour® Capillary Column. Injections (3 μL), made with a Hewlett Packard 6890 series automated injector, were splitless with the purge resuming after 1 min. The carrier gas was N_2 at 2 mL / min with a head pressure of 12 psi. The oven temperature program was 50°C for 1 min followed by 5°C / min to 290°C. The injector temperature was 250°C and the detector temperature 300°C. Peaks which had been identified as pure by GC / MS were quantified using octadecane (C18) as an internal standard and a FID response factor of one was assumed. This assumption was the usual laboratory practice for boronia reporting.

Kovats' Indices

Previous methods used for analysis of boronia extracts used a Hewlett Packard HP1 column. When GC / MS equipment was used that required the use of a Varian column for the first time it was necessary to identify volatiles on the basis of both

mass spectrometric libraries and Kovats' indices. The calculations of Kovats' indices were based on the elution times of a series of hydrocarbon standards ranging from n-C9 to n-C19. The Kovats' indices data is presented in **table 5.1** in comparison to the indices previously calculated by Weyerstahl *et al.* (1995).

Analyte	**Kovats' Index[1]**	**Kovats' Index[2]**
benzyl alcohol	1010	1008
Linalool	1085	1088
7-hydroxy hotrienol	1165	1172
8-hydroxy linalool	1333	1341
7,11-epoxymegastigm-5(6)-en-9-one	1550	1556
3-oxo-5,6-dihydro-β-ionone	1570	1584
unknown C-13 norisoprenoid (MW= 210)	1597	
3-hydroxy-5,6-dihydro-β-ionone	1609	1617(t,c) / 1624(t,t)
methyl diepicucurbate	1625	1637
4-hydroxy-β-ionone	1629	1642
3-hydroxy-β-ionone	1651	1663
methyl cucurbate	1663	1674
methyl epicucurbate	1667	1684

Table 5.1 Comparison of calculated Kovats' indices using a Varian FactorFour® Capillary Column (Kovats' Index[1]) with data published by Weyerstahl *et al.*, (1995) (Kovats' Index[2])

5.3 Results and Discussion

Boronia glycosides, in the form of a purple extract, were isolated from marc using XAD-2 chromatography following solvent extraction with methanol. This extract was then tested with β-D-glucosidase and AR 2000, a broad spectrum pectinase commonly used for release of glycosides in wine and grape juice (Schneider *et al.*, 2001; Cabaroglu *et al.*, 2003; Tamborra *et al.*, 2004), in order to confirm the presence of glycosidic conjugates in boronia. Solvent extracts of the hydrolysates were initially analysed using GC / MS in order to identify the released volatiles. The data is presented in **figure 5.1** and identification was on the basis of mass spectrometric libraries and Kovats' index data (see **Table 5.1**).

The HPLC / MS data, presented in **figures 5.2 and 5.3**, showed the impact of pectinase and β-D glucosidase treatments on the release of volatiles from the proposed glycosidic conjugates in the XAD-2 extract. Enzymatic hydrolysis techniques combined with measurement of the volatiles released are routinely used as an indirect quantitation of glycosidic compounds in plants because of the complexity of the extracts (Schneider *et al.*, 2001). Hence the data obtained in this study confirmed the presence of glycosidic conjugates in boronia through:

(1) The release of volatiles following both enzymatic treatments.

(2) The differential effects between β-D-glucosidase and pectinase treatments.

In relation to the second point the data provided additional evidence for the assignments of glycosidic groupings outlined in chapter 4. The pectinase treatment impacted all of the glycosidic groupings and as would be expected the β-D-glucosidase treatment had no effect on the proposed malonyl glycosides.

The data relevant to C-13 norisoprenoid and cucurbate glycosidic conjugates is represented in **figure 5.2** and indicated that treatment with the pectinase resulted in almost complete disappearance of these compounds. A more limited impact was observed with β-D-glucosidase with no impact on the putative malonyl glycosides and a strong effect on the glycosides of C-13 norisoprenoids. Similarly the putative malonyl glycosides of monoterpenols and monoterpenediols (see **figure 5.3**) disappeared following pectinase treatment. No impact on these compounds was

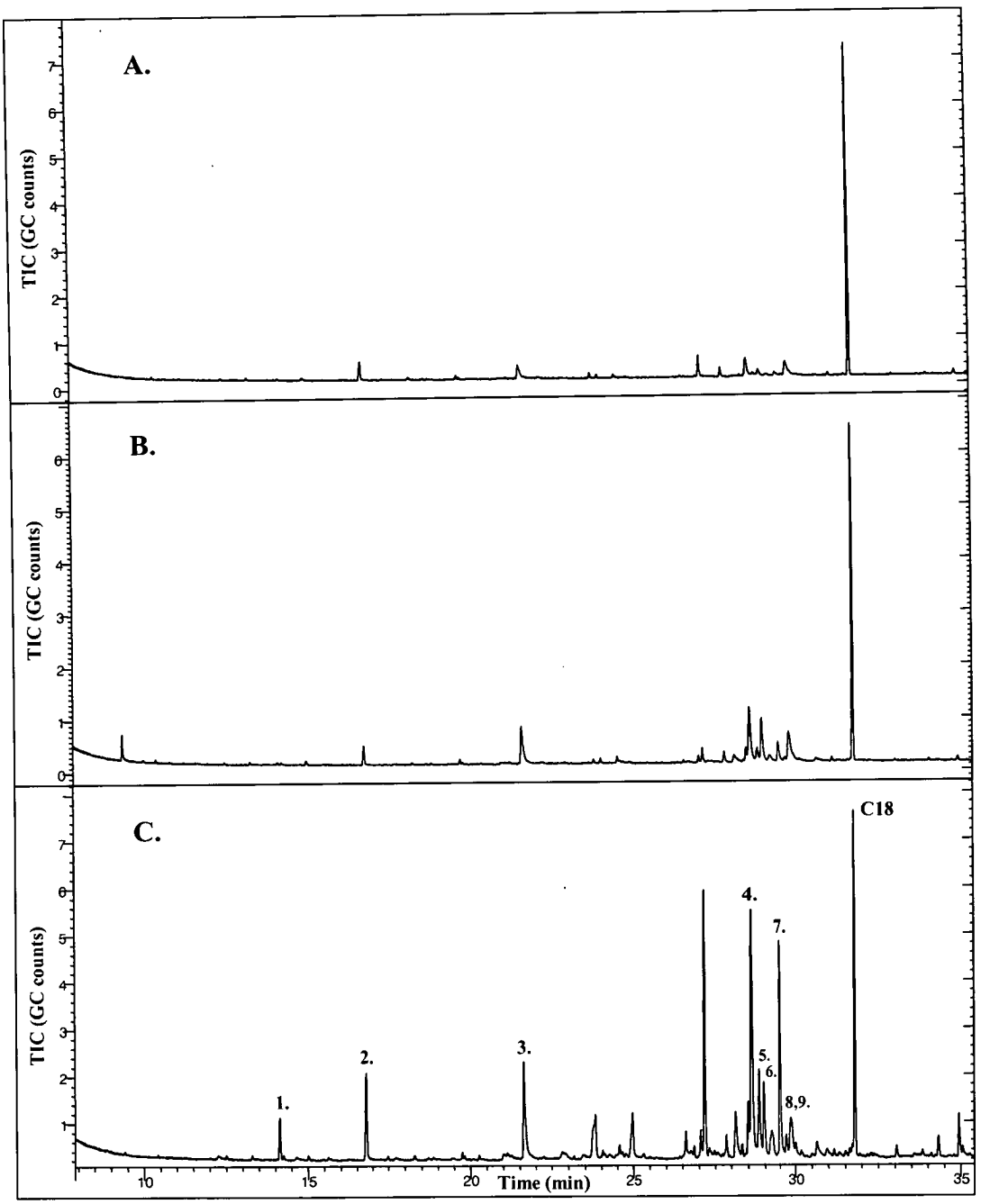


Figure 5.1 GC /MS TIC chromatograms showing the effect of different enzyme treatments on the release of volatiles from a glycosidic extract. **A.** Control; **B.** β-D-glucosidase; **C.** Pectinase, AR 2000.
Key labelled volatiles are: **1.** Linalool; **2.** 7-Hydroxy hotrienol (3,7-dimethylocta-1,5-diene-3,7-diol); **3.** 8-Hydroxy linalool (3,7-dimethylocta-1,6-diene-3,8-diol); **4.** 3-Hydroxy-5,6-dihydro-β-ionone (3-hydroxymegastigm-7-en-9-one); **5.** Methyl-2,3-di-*epi*-cucurbate; **6.** 4-Hydroxy-β-ionone; **7.** 3-Hydroxy-β-ionone; **8.** Methyl cucurbate; **9.** Methyl 3-*epi*-cucurbate.

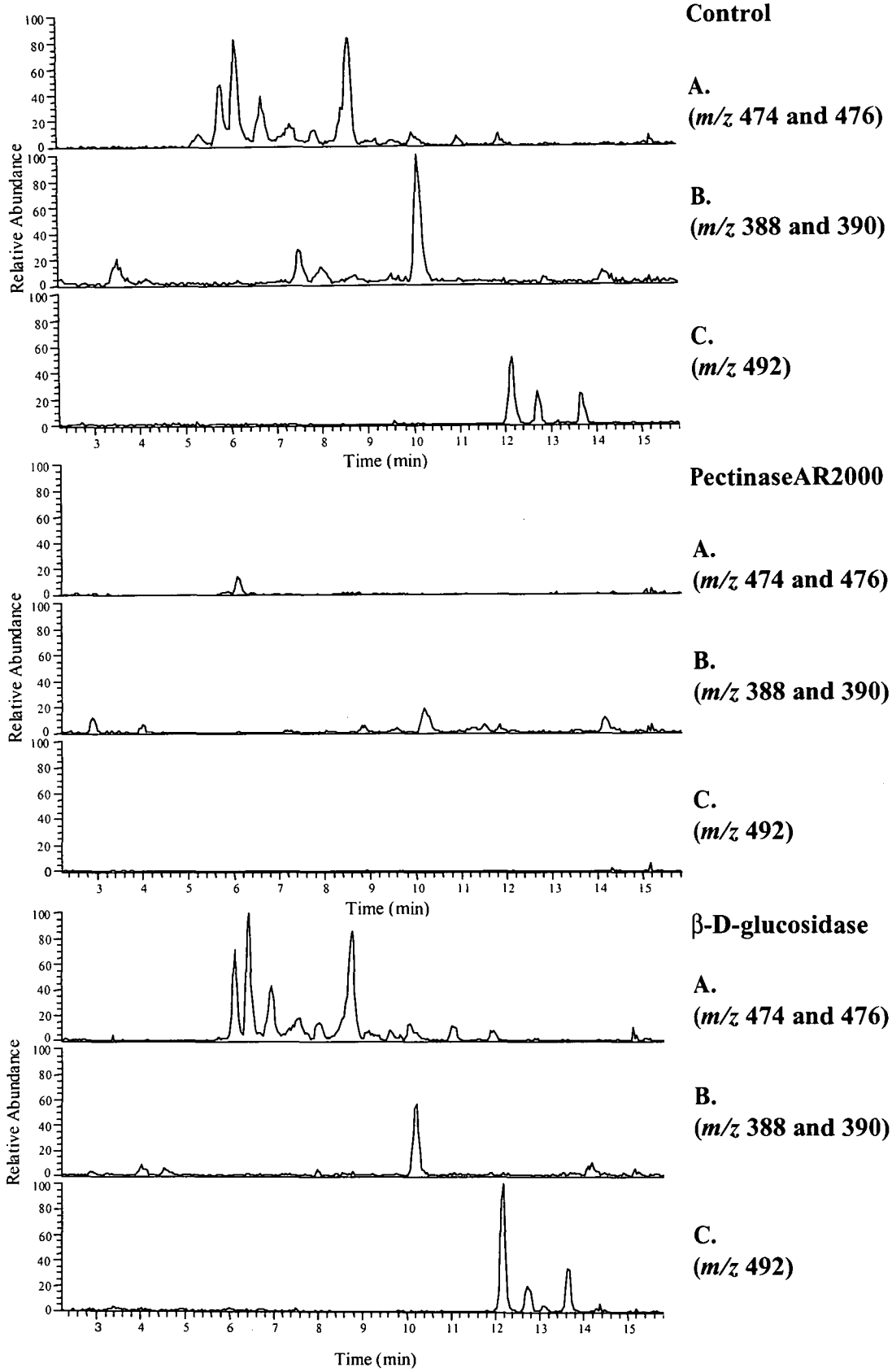


Figure 5.2 Mass chromatograms of $[M+NH_4]^+$ ions showing the effect of different enzyme treatments on **A.** malonyl glycosides of C-13 norisoprenoids, **B.** glycosides of C-13 norisoprenoids, and **C.** malonyl glycosides of cucurbates.

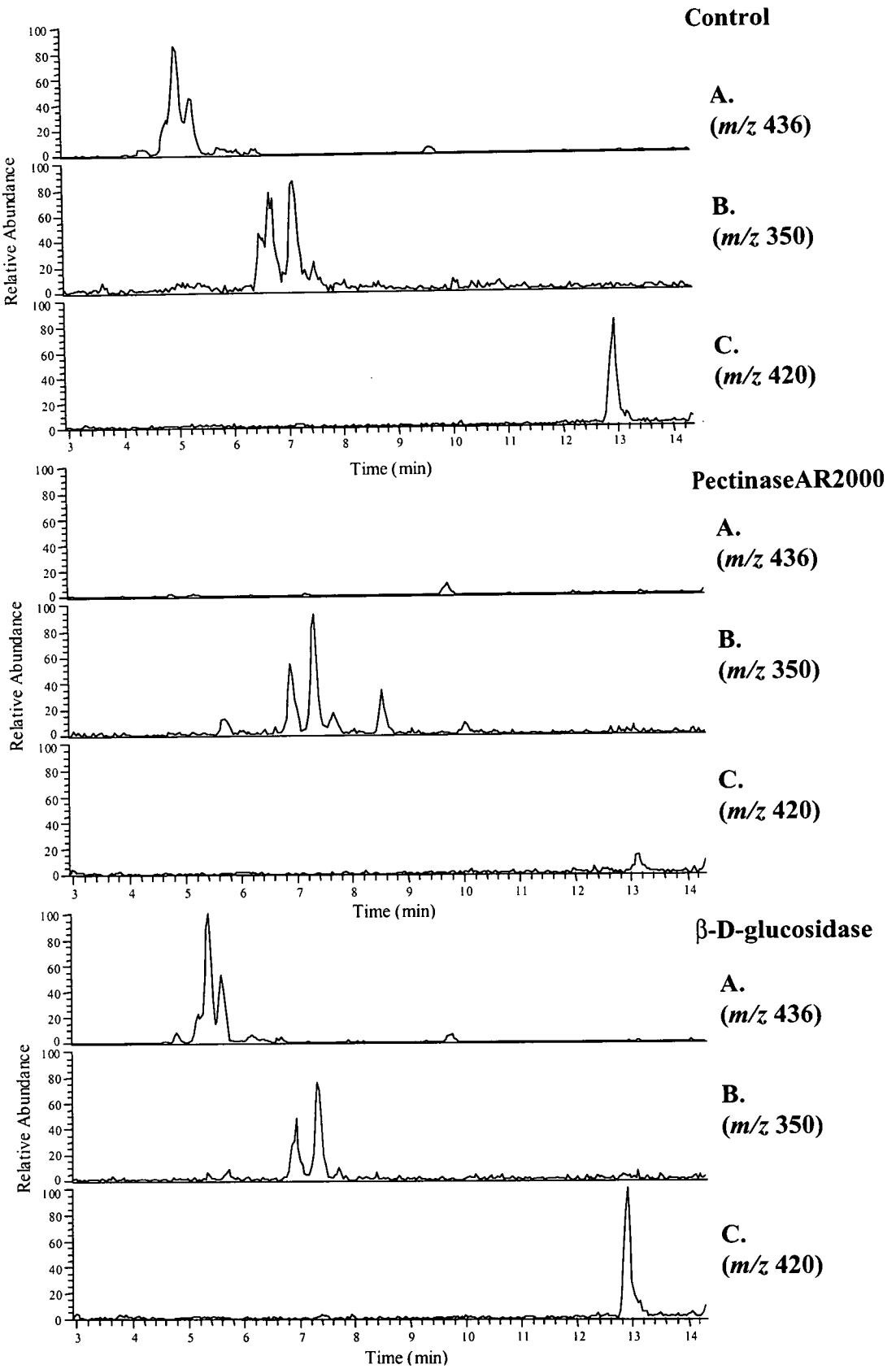


Figure 5.3 Mass chromatograms of $[M+NH_4]^+$ ions showing the effect of different enzyme treatments on **A.** malonyl glycosides of monoterpendiols, **B.** glycosides of monoterpenediols, and **C.** malonyl glycosides of monoterpenols.

observed after treatment with β-D-glucosidase. However a more limited impact was observed with regard to the grouping proposed as simple glycosides of monoterpenediols. This allowed speculation that some of the peaks proposed as glycosides of monoterpenediols were artefactual. However there are clear increases in monoterpenediols following treatment with β-D-glucosidase that corresponded to changes in the HPLC profile for this grouping, as evidenced by the GC / MS and GC / FID data.

The GC / FID data, presented in **table 5.2**, showed the effect of the enzyme treatments on benzyl alcohol, a range of hydroxylated monoterpenes, C-13 norisoprenoids, and cucurbate volatiles. The data assumed an FID response for analytes which is equivalent to octadecane (the internal standard). Both the AR 2000 and β-D-glucosidase treatments resulted in increases in the 13 measured volatiles with higher increases observed for the pectinase treatment. This indicated that the volatiles measured here are bound as both simple glycosides and putative malonyl glycosides. The differences between the control groups (A and B), testing the effect of the incubation alone (16 h at 40°C versus –20°C), were comparatively minimal.

Hydroxylated monoterpenes and C-13 norisoprenoids have been widely reported in the literature as products of conjugate hydrolysis in wine (Cabaroglu *et al.*, 2003) and three monoterpenols were observed to increase during the enzyme treatments reported here. They were linalool, 7-hydroxy hotrienol and 8-hydroxy linalool. Linalool was not detected in the control samples whilst treatment with AR 2000 resulted in the largest increase with the data showing a 7 fold greater concentration than that obtained with the β-D glucosidase treatment. The concentrations of two monoterpenediols, 8-hydroxy linalool and 7-hydroxy hotrienol, were observed to increase following the treatments. The latter compound is considered to be a precursor of hotrienol when hydrolysed under mild acidic conditions (Williams *et al.*, 1980).

Hydroxylated C-13 norisoprenoids were also observed to increase during the enzyme incubations. The largest increase was observed for 3-hydroxy-5,6-dihydro-β-ionone

through treatment with the pectinase. This compound increased from 4.3 to 34.6 μg / 10 mg extract during pectinase treatment which was three times greater than the increase observed with β-D-glucosidase treatment. A larger increase relative to the control was observed for 3-hydroxy-β-ionone which increased from 1.3 to 28.4 μg / 10 mg extract through treatment with the pectinase. This increase was six times greater than that observed through the glucosidase treatment. Less difference between the enzyme treatments was observed for 4-hydroxy-β-ionone with an increase from 2.1 to 12.7 μg / 10 mg extract apparent following the pectinase treatment.

Analyte	**Control A**	**Control B**	**Pectinase AR 2000**	**B-D-glucosidase**
benzyl alcohol	0.30 ± 0.003	0.62 ± 0.09	3.84 ± 0.07	1.77 ± 0.03
Linalool	not detected	not detected	7.17 ± 0.04	1.06± 0.02
7-hydroxy hotrienol	2.01 ± 0.03	3.00 ± 0.03	10.95 ± 0.08	3.56 ± 0.02
8-hydroxy linalool	5.86 ± 0.17	6.62 ± 0.13	21.05 ± 0.15	13.27 ± 0.12
7,11-epoxymegastigm-5(6)-en-9-one	not detected	not detected	2.83 ± 0.03	1.12 ± 0.06
3-oxo-5,6-dihydro-β-ionone	0.85 ± 0.02	0.83 ± 0.02	3.11 ± 0.12	1.31 ± 0.03
unknown C-13 norisoprenoid (MW= 210)	not detected	not detected	15.27 ± 0.11	4.77 ± 0.03
3-hydroxy-5,6-dihydro-β-ionone	4.34 ± 0.05	4.37 ± 0.07	34.63 ± 0.18	11.10 ± 0.06
methyl diepicucurbate	not detected	not detected	10.28 ± 0.20	2.31 ± 0.09
4-hydroxy-β-ionone	2.10 ± 0.02	1.93 ± 0.12	12.74 ± 0.13	8.99 ± 0.07
3-hydroxy-β-ionone	1.33 ± 0.03	1.23 ± 0.06	28.37 ± 0.17	4.52 ± 0.04
methyl cucurbate	not detected	not detected	3.57 ± 0.03	1.36 ± 0.04
methyl epicucurbate	not detected	not detected	4.00 ± 0.04	1.28 ± 0.12

Table 5.2 Effect of enzyme treatment on release of volatiles from a glycosidic extract. The results, expressed as μg component (octadecane equivalent) / 10 mg XAD-2 extract, are the means of 6 replicates and are presented as mean ± standard error.

The cucurbates were not detected in the control treatments with the highest increase to 10.3 μg / 10 mg extract observed for methyl diepicucurbate following treatment with the pectinase. Other compounds observed to increase through the enzyme incubations were benzyl alcohol (which has the capacity to form a glycosidic conjugate), 7,11-epoxymegastigm-5(6)-en-9-one and 3-oxo-5,6-dihydro-β-ionone.

Insight into some of the changes observed in this study might be explained through a consideration of the enzymes that make up a pectinase preparation. Commercial pectinases are generally regarded as complex enzyme mixtures suitable for a range of purposes within the food industry. In addition to the release of flavour and aroma products through glucosidase activity in grape wine (Tamborra *et al.*, 2004) and other food products (Fundira *et al.*, 2002), the multiple enzymatic constituents of pectinases provide rationales for both the presence of oxidation products and the extensive release of volatiles from the malonyl glycosides in boronia.

The primary use of commercial pectinases is for improvements in yield, turbidity, and processing time in the manufacture of fruit juices through the action of enzymes including pectinesterase, endo-polygalacturonase, and endo-pectinlyase (Martino *et al.*, 1994). Additionally, the β-D-glucosidase activity in pectinases has the potential to contribute to colour modification of fruit juice and wine through activity affecting anthocyanins (Martino *et al.*, 1994). However, other enzymes present in pectinases are thought to include a broad spectrum of glucosidases, oxidases (Fundira *et al.*, 2002), and esterases (Roscher *et al.*, 1997).

The studies by Roscher *et al.*, (1997) with regard to stability of 2,5-dimethyl-4-hydroxy-3(2H)-furanone 6'-O-malonyl-β-D-glucopyranoside found this malonyl glycoside to be less stable in acidic conditions than the corresponding β-D-glucoside. This contrasted to the compounds resistance to cleavage by β-glucosidase. Enzymatic hydrolysis of this compound was achieved with a commercial pectinase preparation (Rohapect D5L). The authors claimed that this is a two-step process resulting from esterase activity followed by glucosidase action.

With regard to oxidase activity in pectinase preparations, Sefton and Williams (1991) provided good evidence that oxidation products of hydroxylated C-13 norisoprenoids were produced through the use of several commercial pectinase preparations (Rohapect C, Rohapect D5L and Novoferm 12). In this study both AR 2000 and β-D-gluosidase activity resulted in increased levels of the C-13 norisoprenoid ketone, 3-oxo-5,6-dihydro-β-ionone. It is highly likely that the higher levels of 3-oxo-5,6-dihydro-β-ionone found following pectinase treatment when compared to treatment with β-D-glucosidase are due to enzymatic oxidation from 3-hydroxy-5,6-dihydro-β-ionone. However this does not explain the increases following treatment with β-D-glucosidase.

In order to assess the viability of a commercial process that involves re-extraction of released volatiles from the marc, some estimates were made relating to overall yield of the volatiles released in this study. Using a typical batch extraction of boronia flowers as a model it was calculated that the yield of β-ionone from flowers was approximately 290 mg / kg flowers. This was used as a reference point for the assessment. Based on a yield of 17.8 mg of XAD-2 extract / g of marc the data on released volatiles presented in this chapter (see **Table 5.2**) equated to approximately 280 mg / kg of marc. The hydroxylated ionones represented approximately 135 mg / kg marc. Overall this data indicated the potential of post harvest hydrolysis processes to obtain compounds with potential aroma and flavour value with an equivalent yield to endogenous β-ionone from fresh flowers.

Chapter 6
Glycosidic Conjugates in Boronia

6.1 Introduction

The methodology developed during the glycoside screening process allowed further experimentation to explore the changes during biological processes. The initial part of this chapter considered relevant differences in glycosidic conjugates due to clonal variation, the stages of flower development in boronia, and between leaves and flowers. Experiments were begun concurrently that considered the potential for a simple marc incubation technique that might result in the release of volatiles. This was based on the observation (personal communication, R. Menary) that boronia marc left in a pile would show signs of fungal growth and begin to emit an aroma that included sensations typically found in boronia extracts. A comprehensive assessment was made of the potential for a simple marc incubation to result in the release of volatiles.

6.2 Materials and Methods

6.2.1 Materials

All reagents were analytical grade and solvents were either redistilled or HPLC grade. Boronia flowers, marc and leaves were obtained from Essential Oils of Tasmania at Kingston or from plants grown at Kingston in Southern Tasmania.

6.2.2 Small-scale Conjugate Extraction Process

Boronia flowers, marc and leaves (0.5 g) were accurately weighed into a polyethylene tube and 2.0 mL of ice cold methanol was added. The marc was then homogenised using an ultraturrex (T25 basic, Ika labortechnik, setting 6), fitted with

an 8 mm head. Samples were then centrifuged (Beckman J2 – 21 M / E, rotor 20.1) at 10, 000 g for 5 min. These steps were performed at 4°C. In some experiments an aliquot of the resultant supernatant (1 mL) was applied to a Waters Sep-Pak C18 cartridge that had been pre-equilibrated with methanol. Following application the cartridges were washed with 1 mL of methanol and the combined eluants were collected and stored at 4°C prior to HPLC / MS analysis. Preliminary tests had ascertained that C18 SPE chromatography did not significantly lower the yields of the glycosidic conjugates.

6.2.3 Extraction of Volatiles

Flower or marc samples were transferred to a conical flask and extracted with petroleum ether according to the method of MacTavish and Menary (1999a). Accordingly, the incubated marc samples were extracted three times with pentane : hexane (80 : 20) by first washing for 120 min followed by two subsequent washes for 60 min each. Samples were then rinsed with 50 mL of solvent. Samples were shaken during the extraction period using a Gallenkamp Cooled Orbital Shaker at 100 rev / min at room temperature. After each extraction the solvent extracts were filtered through cotton wool, combined, and stored at 4°C prior to solvent removal with a rotary evaporator. Each sample was evaporated to dryness at 35°C and then subjected to a final rotary evaporation drying period at 50°C for 5 min.

6.2.4 HPLC / MS

Methanol extracts of boronia were analysed for changes in glycosidic conjugates using HPLC / MS. Analysis was performed with a Waters 2690 HPLC and Finnigan LCQ. The HPLC was equipped with a Waters Novapak C18 3.9 mm x 150 mm column and an Alltech Econosphere C18 guard cartridge.

HPLC Program A

The initial conditions were 90% water / 10% methanol changing to 10% water / 90% methanol at 25 min. The flow rate was 0.8 mL / min and the injection volume 5 μL.

HPLC Program B

The initial conditions were 30% methanol / 70% 0.1 M ammonium acetate (pH 6.38) with a linear gradient to 70% methanol / 30% 0.1 M ammonium acetate (pH 6.38) at 40 minutes. The flow rate was 0.8 mL / min and the injection volume 5 µL.

Mass Spectrometry

Typical MS conditions were APCI source; APCI Vapouriser Temperature: 460°C; Capillary Temperature: 170°C; Sheaf Gas Flow: 60 psi; Capillary Voltage: 41 V. Full scan analysis was usually over the range *m/z* 150 – 750 at 1 cycle / s and Finnigan Navigator software was used to generate and analyse the data.

6.2.5 GC / MS Analysis

Petroleum ether extracts, obtained using the method of MacTavish (1995), containing released volatiles were analysed using a Hewlett Packard 5890 Gas Chromatograph and Hewlett Packard 58790B Mass Selective Detector. The GC was fitted with a 25 m x 0.32 mm i.d. x 0.17 µm film thickness HP1 column. Injections were made in splitless mode. The carrier gas was helium with a head pressure of 10 psi. The initial oven temperature was 50°C, held for 2 min, and then rising to 280°C at 10°C / min. Scanning was from *m/z* 35 to *m/z* 350 at 1.9 scans per second in electron ionisation mode (70 eV). Peaks were identified by a combination of commercial (NIST, version 2.0) and in-house databases. Mass chromatograms of diagnostic ions were extracted from the data and the TIC calculated on the known percentage from un-contaminated spectra.

6.2.6 GC / FID

Marc obtained from a commercial scale trial was analysed using the method of MacTavish and Menary (1997). Accordingly a Hewlett Packard 5890 Series II GC equipped with a Flame Ionisation Detector (FID) and an HP-1 cross linked methyl silicon gum column (30 m x 0.2 mm i.d., 0.33 µm film thickness) was used for analysis. The carrier gas was N_2 at 2 mL / min with a head pressure of 12 psi and a

split ratio of 1 : 50. The oven temperature program was 50°C for 1 min followed by 10°C / min to 250°C. The injector temperature was 250°C and the detector temperature 280°C. Peaks which had been identified as pure by GC / MS were quantified using octadecane (C18) as an internal standard and a FID response factor of one was assumed. This assumption was the usual laboratory practice for boronia reporting.

6.2.7 GC / Olfactometry

Marc samples were analysed using a Hewlett Packard 5880A series Gas Chromatograph equipped with a sniffing port. The methodology was the same as described in section 6.2.6 with 50% of the column flow directed to the sniffing port. The olfaction times were recorded on a stopwatch and where possible compared to the FID peak times from the chromatogram.

6.2.8 Clonal Variation

Flowers from the four different boronia clones (3, 5, 17 and 250) routinely grown for commercial extraction were extracted with methanol. The extracts were analysed for differences in the levels of glycosidic conjugates of C-13 norisoprenoids, cucurbates and monoterpenes using the extraction and HPLC / MS screening processes developed in chapter 4.

6.2.9 Flower Development and Leaf studies

Flowers and leaves from clones 22, D3 and D4 were examined for variation between leaves and flowers and between leaves and buds. Flowers were selected for analysis according to the protocol described in **section 3.2.2**. Leaves were selected for study which were adjacent to either the buds (stage 1 and 2) or open flowers (stage 4).

6.2.10 Post Harvest Incubation Experiments

Small-scale Trials

A portion of boronia marc that had been newly removed from the commercial extractor was arranged into a pile of approximately 1 m diameter and 15 cm in height. Samples were removed for analysis at 0, 3 and 5 days after beginning the incubation process and analysed by HPLC / MS to detect changes in glycosidic conjugates of C-13 norisoprenoids. Note that with regard to data analysis the early HPLC methodology used in this section did not allow analysis of the full suite of glycosidic conjugates described in chapter 4. The early focus of this study was on C-13 norisoprenoids and for this sub-section analysis of selected malonyl glycosides and simple glycosides of these compounds were used for assessing the efficacy of the incubation process. The results for glycosides and rutin are presented as the percentage change in peak areas.

Ten Day Trial

Boronia marc which had been newly removed from a commercial extractor was arranged into three piles on plastic sheets covering a concrete floor. The piles were approximately 1.5 m in width and 20 cm deep. Two samples (approx. 500 g) were taken from randomly selected places in each of the three piles at 0, 2, 4, 6, 8 and 10 days following initiation of the incubation period. This resulted in 6 replicates which were then analysed by GC / MS for release of volatiles.

6.2.11 Commercial Trial

Marc Incubation

A commercial trial was conducted on marc from clone 3 flowers. The marc used in this trial was obtained following extraction of a 600 kg batch of flowers that had been processed in the normal commercial manner by Essential Oils of Tasmania. The marc was spread on sheets of plastic on the factory floor and left to incubate for a period of exactly four days prior to re-extraction. The area over which the marc was spread measured approximately 6 x 3 m. The incubated marc weighed 432 kg

prior to re-extraction and the re-extraction process was performed according to the usual petroleum ether extraction procedure used by Essential Oils of Tasmania.

A partially rotary-evaporated concentrate, representing all of the material re-extracted, was provided by Essential Oils of Tasmania to the University laboratory. The remaining solvent was removed from the extract to yield 1.40 kg of marc extract. The clone 3 marc extract was analysed by GC / MS and GC / FID.

Organoleptic Testing

Samples of marc were analysed by GC / Olfactometry (see **section 6.2.5**) and aroma dilution analysis. For the dilution analysis, marc samples were dissolved in ethanol to form a 1% solution. These solutions were then used for organoleptic testing through the addition of three drops of each solution to 100 mL of distilled water in a wine glass prior to aroma evaluation.

6.2.12 Temperature Monitoring

The temperature of marc samples during incubation was monitored with Hobo®-Temp temperature data loggers (Onset Computer Corp). Data loggers were placed at a central depth in the piles and a minimum of 20 cm from the edge of the pile.

6.2.13 Direct Probe Mass Spectrometry of Boronia Marc

A sample of clone 3 marc was analysed using a Kratos Concept ISQ in electron ionisation mode (70 eV) with a direct insertion probe. The spectra were averages of the distillation profile.

6.3 Results and Discussion

6.3.1 Clonal Variation of Conjugates

Differences in the relative levels of glycosidic conjugates in boronia clones were examined in order to assess their potential with regard to possible outcomes from post-harvest incubation processes. Accordingly, the commercially available clones 3, 250, 17 and 5 were examined for six different conjugate types and the results are presented in **table 6.1**.

Conjugate type	Clone 3	Clone 250	Clone 17	Clone 5
Malonyl glycosides of C-13 norisoprenoids	129.3 ± 5.6	87.1 ± 9.1	76.2 ± 3.7	94.6 ± 10.2
Mono-glycosides of C-13 norisoprenoids	34.0 ± 0.6	38.6 ± 2.6	26.6 ± 1.3	46.9 ± 6.9
Malonyl glycosides of Cucurbates	47.2 ± 1.1	37.0 ± 4.4	33.2 ± 0.3	40.7 ± 4.8
Malonyl glycosides of monoterpenediols	23.0 ± 2.1	34.2 ± 2.3	36.2 ± 1.0	18.3 ± 1.5
Mono-glycosides of Monoterpenediols	6.7 ± 0.1	12.3 ± 1.7	11.2 ± 0.1	8.5 ± 0.7
Malonyl glycosides of Monoterpenols	18.2 ± 1.2	35.2 ± 2.1	24.9 ± 0.2	14.7 ± 1.2

Table 6.1 Clonal variation in conjugate levels. The data representing the means and standard errors of three replicates is relative peak area (peak area / standard area) / g marc.

Clone 3 had the highest levels of malonyl glycosides of C-13 norisoprenoids and cucurbates whilst clone 5 had the highest levels of mono-glycosides of C-13 norisoprenoids. In contrast, clone 17 had the lowest levels of all glycosidic

conjugates of C-13 norisoprenoids and cucurbates. The highest relative difference between clones in any one conjugate group was for malonyl glycosides of monoterpenols. In that conjugate class the value for clone 5 flowers represented 42% of the amount found in clone 250 flowers. Following this experiment clone 3 flowers were selected for more extensive trials of a simple incubation process (see section 6.3.4). This was on the basis of both flower availability and the higher levels of glycosides of C-13 norisoprenoids in this clone.

6.3.2 Changes during Flower Development

Boronia flowers and buds from clone 3 flowers were examined for changes in glycoside content during flower development. The results, presented in **table 6.2**, showed that all of the glycoside classes identified in **chapter 4** increased during flower development. All classes of glycosides showed the largest increases between bud opening and the open flower stage.

The main differences observed between the different classes of glycosides were with regard to their initial appearance. The changes observed here can be related to the results from **chapter 3** where changes in β-ionone, C-27 apocarotenoids and C-40 carotenoids during flower development were reported. The results for the C-13 norisoprenoid glycosides showed a similar overall pattern of increase to that observed for the C-27 apocarotenoid increases described in chapter 3 with the exception that trace amounts of malonyl glycosides of C-13 norisoprenoids were detected in buds at stage 1 of flower development. This indicated that low levels of xanthophyll cleavage occurred during that early stage of flower development.

Malonyl glycosides of cucurbates were not detected at stage 1 of flower development. However the monoterpenols showed a more complex pattern of early appearance and the results indicated that the biosynthesis of monoterpenes occurred earlier than the C-13 norisoprenoids. Whilst no simple glycosides of monoterpenediols were detected in small buds, malonyl glycosides of both monoterpenols and monoterpenediols were present at the early stage. This may be reflective of the different routes for biosynthesis of the two groups of compounds.

Clearly the enzymes for biosynthesis of the malonyl glycosides of alcohols are present in the small bud stage. It is likely however that the biosynthesis of the carotenoid cleavage enzyme with enough activity for the measurement of cleavage products and their glycosides does not begin until just before flower opening.

Conjugate type	Stage 1	Stage 2	Stage 3	Stage 4
Malonyl glycosides of C-13 norisoprenoids	Trace	4.43 ± 0.38	6.01 ± 0.56	16.33 ± 1.12
Simple glycosides of C-13 norisoprenoids	not detected	3.58 ± 0.34	4.49 ± 0.28	7.17 ± 0.56
Malonyl glycosides of cucurbates	not detected	1.13 ± 0.07	2.54 ± 0.26	7.80 ± 0.47
Malonyl glycosides of monoterpenediols	2.71 ± 0.22	3.97 ± 0.25	5.37 ± 0.40	13.71 ± 0.75
Glycosides of monoterpenediols	not detected	0.32 ± 0.01	0.64 ± 0.03	2.30 ± 0.06
Malonyl glycosides of monoterpenols	0.56 ± 0.04	1.90 ± 0.09	3.04 ± 0.21	10.27 ± 0.61

Table 6.2 Changes in glycosides during flower development. The data is expressed as means ± standard errors of three replicates and is defined as (peak area / standard area) / sample.

The appearance of monoterpenes at the early stages of flower development is supported by the results reported by MacTavish (1995) who found that the hydrocarbon monoterpenes β-pinene and limonene are present at the earliest stage of flower development. For comparative purposes this overlap in the stages used for the two studies needs to be explained. Stage 1 (small bud stage) in these studies equated to stages 1 through 3 inclusively (very small, small and medium size buds) of the MacTavish studies. The level of limonene was reported (MacTavish, 1995) as highest at the very small bud size (Stage 1 - as defined by MacTavish) whilst the level of β-pinene was highest in the medium sized buds (Stage 3 – as defined by

MacTavish). This contrasted in both studies with the observation that β-ionone first appeared at the large bud stage (defined similarly by both studies).

The organoleptic characteristics of extracts obtained at different stages of flower development (MacTavish, 1995) were reflective of the differences observed for the first appearance of monoterpene and C-13 norisoprenoid glycosides. Floral elements that might be associated with norisoprenoids appeared first at the open flower stage whereas aroma sensations consistent with hydrocarbon monoterpenes were apparent in very small buds. Overall the results demonstrated that the capacity exists in boronia for the biosynthesis of malonyl glycosides and that by inference monoterpene alcohols are present in small buds. However there is no obvious reason for the lack of early appearance of simple monoterpene glycosides.

6.3.3 Glycoside Profiles in Leaves

The possibility that glycosides in flowers were synthesised in leaves prior to transport to the flowers was examined in a qualitative study. A preliminary experiment was conducted to compare the differences between flowers, buds and leaves. Given that subtending leaves and the inflorescence have both a common connection through the vascular system and a close sink-source relationship (Wahid *et al.*, 2003), they were considered likely to provide the most useful comparative data. The study included leaves which were either adjacent to flowers or buds. Distinct differences were observed between flowers and leaves. These differences, in clone 22 plants, are depicted for malonyl glycosides of C-13 norisoprenoids. The results presented in **figures 6.1 and 6.2** showed that leaves adjacent to flowers have a different chemical profile to the flowers. The differences in the profiles were examined in detail through comparison of the individual glycosides corresponding to hydroxylated ionones (*m/z* 474 for $[M+NH_4]^+$) and hydroxylated dihydro ionones (*m/z* 476 for $[M+NH_4]^+$).

The malonyl glycosides of hydroxy ionones (**figure 6.1 trace B** for both flowers and leaves) showed a similar pattern of appearance with different levels of intensity for

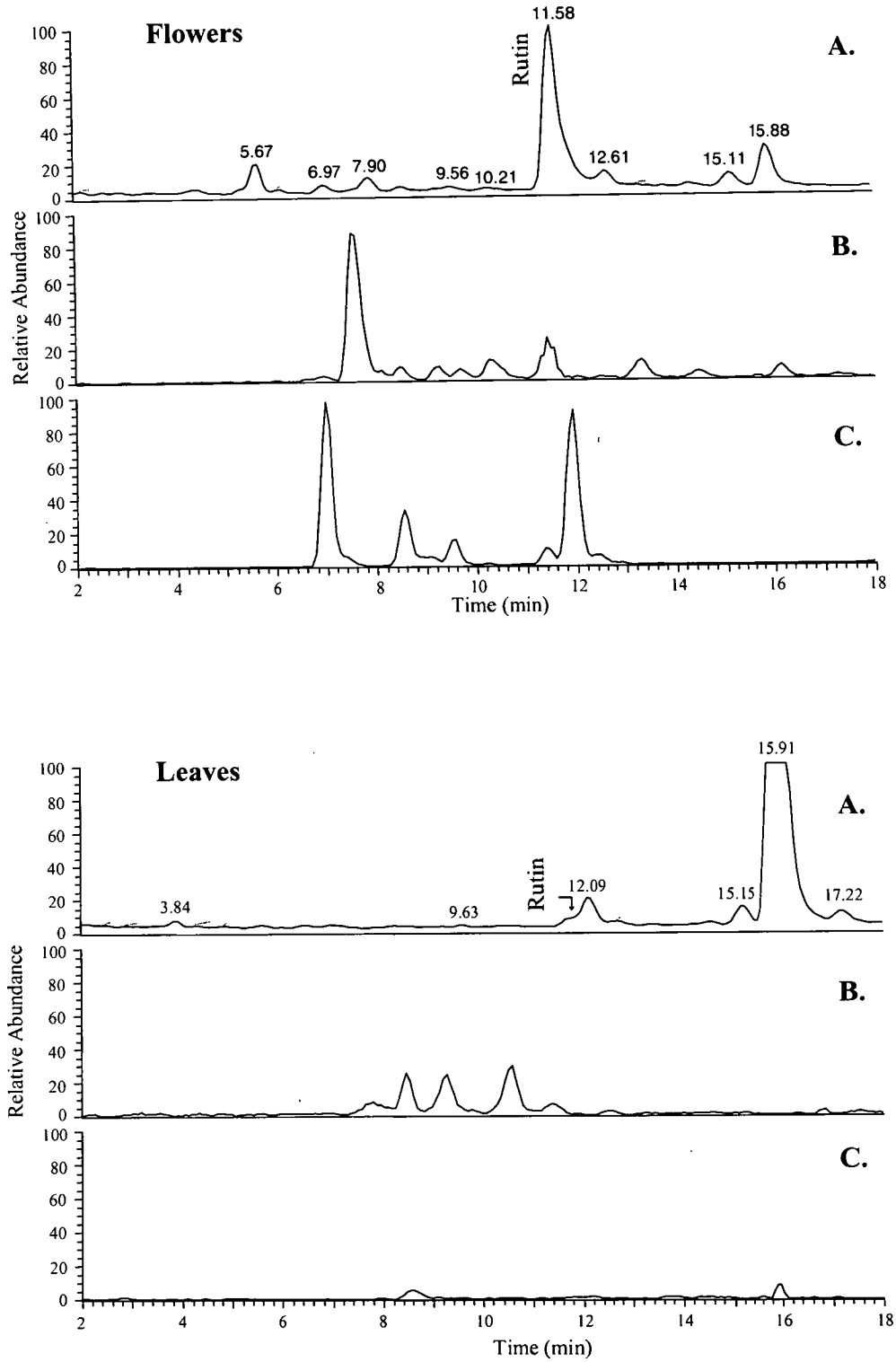


Figure 6.1 Profiles showing the distribution of C-13 norisoprenoid malonyl glycosides in clone 22 boronia flowers and leaves adjacent to flowers.
A. TIC.
B. Mass chromatogram of *m/z* 474 ions ($[M+NH_4]^+$ for $C_{13}H_{20}O_2$ norisoprenoids)
C. Mass chromatogram of *m/z* 476 ions ($[M+NH_4]^+$ for $C_{13}H_{22}O_2$ norisoprenoids)
Relevant peaks are between 6 and 13 mins.

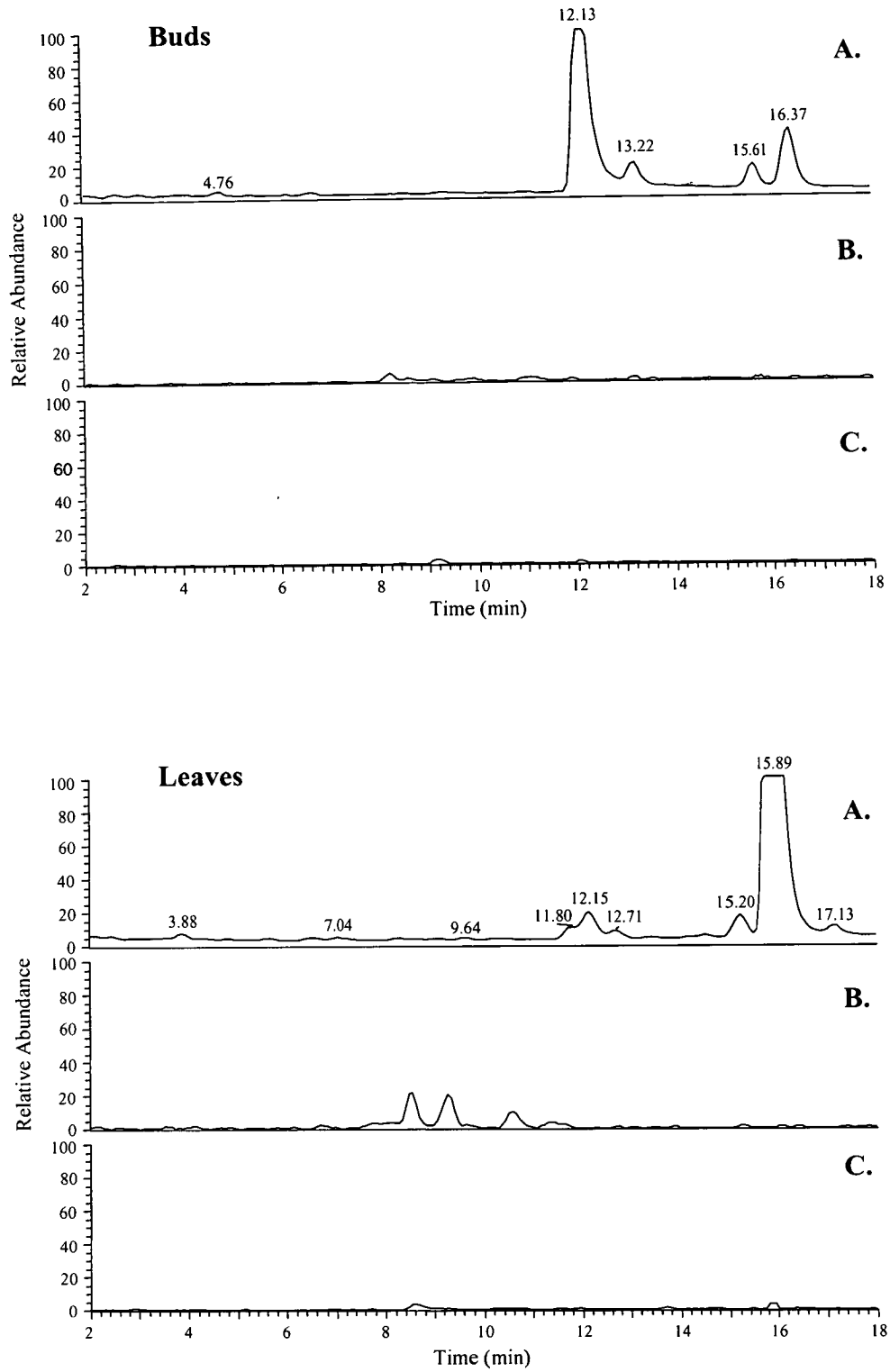


Figure 6.2 Profiles showing the distribution of malonyl glycosides in clone 22 boronia buds and leaves adjacent to buds.
A. TIC.
B. Mass chromatogram of *m/z* 474 ions ($[M+NH_4]^+$ for $C_{13}H_{20}O_2$ norisoprenoids)
C. Mass chromatogram of *m/z* 476 ions ($[M+NH_4]^+$ for $C_{13}H_{22}O_2$ norisoprenoids)
Relevant peaks are between 6 and 13 mins.

each of the peaks. However the pattern for hydroxy dihydro ionone glycosides indicated there were distinct differences between leaves and flowers. The two main peaks in the flower profile appeared to be absent in the leaf profile. The leaves adjacent to buds showed a similar pattern of appearance for the glycosides to that obtained from leaves adjacent to flowers and as observed in the flower development study only minimal levels of these compounds were observed in the buds. Further studies with other clones indicated similar results. The monoterpene glycosides showed an even greater difference between flowers and leaves in three different clones. In clones 22 and D4 no glycosides of monoterpene alcohols that were apparent in the flowers were present in the leaves adjacent to either flowers or buds. In clone D3 peaks equating to some glycosides of monoterpene alcohols were present at an order of magnitude lower level.

Previously, Winterhalter and Skouroumounis (1997) detailed a rationale, attributed to Watanabe *et al.* (1993), proposing that glycosides of volatile compounds were initially synthesised in leaves and transported to flowers prior to hydrolysis through the action of flower glycosidases. These studies do not support this rationale for the following reasons. Firstly several glycosides found in the flowers were not observed in leaves. Additionally the patterns in the leaves adjacent to either flowers or buds showed similar patterns and levels whilst there was an ongoing increase in the levels of these compounds in flowers and buds during flower development. This later observation is not suggestive of a relationship between the leaves and inflorescences. Note that the scale of attenuation for leaves and bud data in **figures 6.1 and 6.2** were matched in corresponding mass chromatograms for comparative purposes. This represented a doubling of sensitivity in comparison to the flowers further emphasising the low levels of these compounds in leaves. The methodology does not allow any comparison between the hydroxy ionone and hydroxy dihydro ionone mass chromatograms without the use of known standards.

A study by Gholami *et al.* (1996) however may have been supportive of the rationale for transport of glycosides from leaves to grape berries. The authors found that glucose bound as glycosides increased in the leaf and petiole during berry development. However levels decreased in the berry over the same period. Differences in glycoside levels between leaves and buds were also studied in Laurel

(Kilic *et al.*, 2005). A range of differences were observed including that damascenone related C-13 norisoprenoids were released from precursors in the buds but were not found in the leaves. Additionally the authors found that monoterpene glycosides were higher in buds than in leaves. However the authors did not report on the changes in glycoside levels during flower development. Many of the recent studies with regard to plant volatiles have focussed on leaves (see **table 1.2** for references) and the potential for creating plant extracts with volatiles released from leaf glycosides in boronia is worth consideration. However, this preliminary study with boronia indicated that leaves have overall lower levels of glycosidic precursors than occur in boronia flowers.

6.3.4 Post Harvest Changes

Small-scale Trials

A series of experiments were conducted to further understand the observations by Menary (personal communication) that, following the extraction process, boronia flowers showed signs of both fungal growth and volatile emission. Initial experiments were conducted during the early stages of method development for the analysis of glycosidic conjugates. Consequently the data presented in **table 6.3** below refers only to measured changes in malonyl and mono-glycosides of C-13 norisoprenoids during a marc incubation experiment conducted over 5 days. The mono-glycosides were reduced by 44% during the first three days with an overall reduction of 82% after 5 days. The malonyl glycosides were reduced more slowly in the initial stages of the incubation with a 29% reduction after three days. The reduction of the malonyl glycosides at five days (85%) was however similar to that which occurred for the mono-glycosides.

As no internal standard was included in this experiment, glycoside changes were compared to changes in the level of rutin over the experimental period. Minimal changes in rutin were observed and the data was used to verify the repeatability of measurements between runs. Identification of rutin was on the basis of a known standard. Further work with this technique included an assessment of volatiles released during the marc incubation and resulted in a more extensive ten-day trial.

Incubation Period (days)	Malonyl glycosides of C-13 norisoprenoids	Simple glycosides of C-13 norisoprenoids	Rutin
0	100% (268.1 ± 1.5)	100% (103.4 ± 3.5)	100% (14578.3 ± 440.4)
3	71.4% (191.9 ± 11.7)	56.4% (58.3 ± 1.0)	90.1% (13135.7 ± 414.6)
5	15.0% (40.3 ± 1.8)	18.1% (18.7 ± 2.1)	93.7% (13656.7 ± 618.8)

Table 6.3 Changes in selected glycosides of C-13 norisprenoids during a simple factory floor incubation of boronia marc. The results are expressed as a percentage of the level present at zero time. The equivalent peak area means and standard errors ($x10^6$) of three replicates are also presented.

Ten-day Trial

Following the small scale marc incubations which demonstrated both the disappearance of conjugates and the appearance of C-13 norisoprenoid and monoterpenol volatiles, a more comprehensive trial was conducted over ten days. Six replicate samples were collected every two days during the incubation period and analysed by GC / MS for the appearance of volatiles, the data for which is presented in **Table 6.4**. There was evidence that several overlapping processes were occurring during the marc incubation. Most importantly, this technique showed promise as a process for increasing the yield of volatiles from boronia as there were clear increases over time in the levels of monoterpenols, cucurbates and C-13 norisoprenoid alcohols. The changes in the levels of monoterpenes and hydroxy ionones is presented graphically for comparative purposes in **figures 6.3 and 6.4** and a detailed consideration follows.

Linalool increased 19 fold from 0.14 mg / kg marc at 0 days to 2.6 mg / kg marc at 4 days with a high level of 2.4 mg / kg marc persisting at 6 days. Subsequently the levels of linalool decreased rapidly to 0.3 mg / kg marc at 10 days. A similar pattern was observed for 8-hydroxy linalool with an increase from 1.2 mg / kg marc to 2.8 mg / kg marc at 4 days. However the changes in 8-hydroxy linalool levels

Incubation Time	0 days	2 days	4 days	6 days	8 days	10 days
Linalool	0.14 ± 0.02	0.13 ± 0.01	2.64 ± 0.59	2.41 ± 0.38	0.54 ± 0.04	0.27 ± 0.01
7-hydroxy hotrienol	0.64 ± 0.03	0.76 ± 0.02	2.03 ± 0.27	6.16 ± 0.27	6.59 ± 0.27	7.06 ± 0.64
8-hydroxy linalool	1.18 ± 0.07	1.95 ± 0.02	2.83 ± 0.11	0.80 ± 0.08	0.42 ± 0.03	0.37 ± 0.02
3-oxo-5,6-dihydro-β-ionone	0.76 ± 0.03	0.94 ± 0.004	1.57 ± 0.10	10.59 ± 0.51	12.23 ± 0.34	4.30 ± 0.59
7,8-dihydro-β-ionone	0.55 ± 0.02	0.73 ± 0.02	6.62 ± 0.18	8.16 ± 0.27	5.31 ± 0.18	1.56 ± 0.15
β-ionone	8.73 ± 0.21	11.08 ± 0.09	4.70 ± 0.28	0.35 ± 0.04	0.44 ± 0.08	0.31 ± 0.01
7,11-epoxymegastigma-5(6)-en-9-one	0.45 ± 0.01	0.77 ± 0.01	2.55 ± 0.37	2.80 ± 0.10	1.51 ± 0.10	0.62 ± 0.07
3-hydroxy-5,6-dihydro-β-ionone	7.16 ± 0.20	8.35 ± 0.05	23.52 ± 3.52	34.73 ± 0.96	27.11 ± 1.80	6.98 ± 0.68
4-hydroxy-β-ionone	1.19 ± 0.04	1.94 ± 0.07	6.19 ± 1.32	2.11 ± 0.13	1.58 ± 0.08	0.61 ± 0.05
3-hydroxy-β-ionone	1.00 ± 0.04	1.47 ± 0.02	3.84 ± 0.66	1.05 ± 0.08	0.71 ± 0.07	0.34 ± 0.02
methyl diepicucurbate	0.67 ± 0.04	1.18 ± 0.03	6.13 ± 1.02	6.06 ± 0.16	3.27 ± 0.31	0.65 ± 0.06
methyl cucurbate and methyl epicucurbate	1.47 ± 0.05	1.89 ± 0.04	7.30 ± 1.10	10.21 ± 0.27	6.55 ± 0.22	1.98 ± 0.29
heptadec-8-ene	7.98 ± 0.20	14.60 ± 0.37	16.24 ± 0.33	16.84 ± 1.26	14.48 ± 1.17	15.61 ± 3.40

Table 6.4 Changes in the concentration of volatiles (mg / kg marc – octadecane equivalent) during ten days of a simple marc incubation. The data, which represents the means and standard errors of six replicates, is derived from mass chromatograms of diagnostic ions. The area for each ion was extracted from the data and the TIC calculated on the known percentage of the ion from un-contaminated spectra.

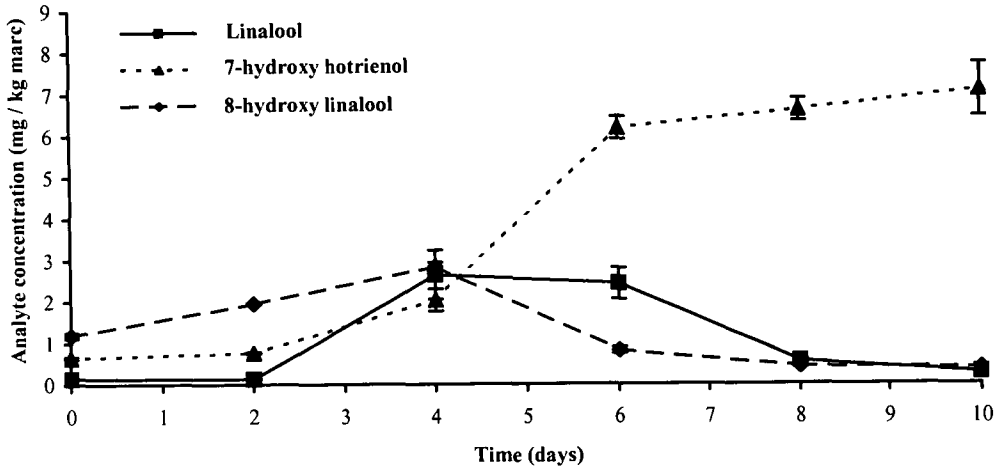


Figure 6.3 Changes in monoterpenol levels during a simple ten-day marc incubation trial. Data represents mean and standard errors of 6 replicates.

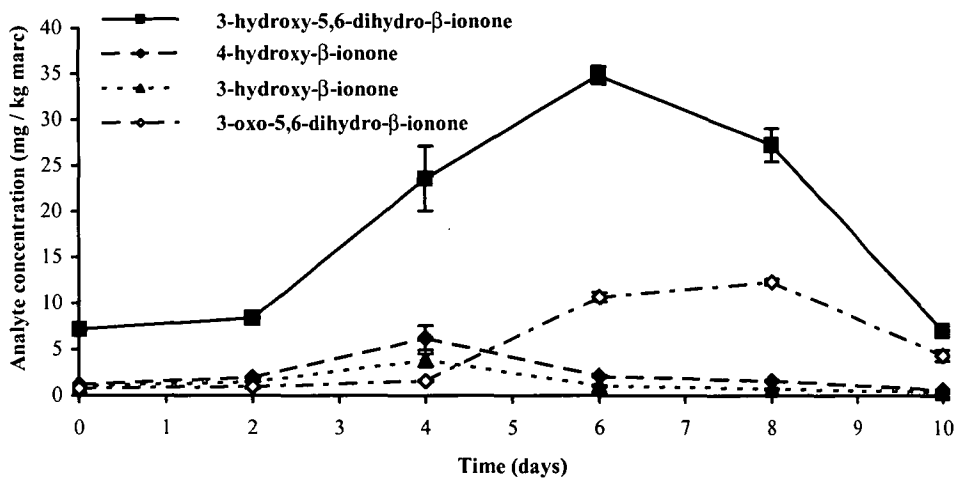


Figure 6.4 Changes in polyoxygenated ionones during a simple ten-day marc incubation trial. Data represents mean and standard errors of 6 replicates.

compared to linalool showed (1) a smaller increase relative to initial levels and (2) an earlier increase with a more rapid decrease after 4 days. The pattern of change observed for 7-hydroxy hotrienol was very different to that observed for the other two monoterpenols measured in this experiment. Levels of this compound increased continuously throughout the experiment by a factor of 11 from 0.6 mg / kg marc to 7.1 mg / kg marc at 10 days.

The differences between the changes in the relative levels of 7-hydroxy hotrienol and the other monoterpenols might be due to ongoing conjugate hydrolysis and / or the absence of further metabolism following release. Evidence exists in the literature for variability in the degradation of monoterpenes and in an experiment comparing the degradation of four different monoterpenols, Misra *et al.* (1996) found that linalool and terpineol degraded more rapidly than plinol and arbanol. The authors also observed that terpineol was degraded more slowly in the presence of glucose. Similar observations were noted by Larroche *et al.* (1995) who observed the absence of β-ionone metabolism in the presence of carbohydrate energy sources. The availability of carbohydrate energy sources may be an important factor with regard to the development of a methodology for release and extraction of bound volatiles and it is probable that the rate of further metabolism of the released compounds occurs more slowly whilst released sugars are present.

The C-13 norisoprenoid alcohols also showed significant increases during the incubation. Three hydroxy ionones (3-hydroxy-5,6-dihydro-β-ionone, 4-hydroxy-β-ionone, and 3-hydroxy-β-ionone) were measured and the changes are presented in **figure 6.4** along with associated changes in 3-oxo-5,6-dihydro-β-ionone. The highest level of the hydroxy C-13 norisoprenoids (relative to C18) was for 3-hydroxy-5,6-dihydro-β-ionone which increased from 7.2 mg / kg marc at zero time to 23.5 mg / kg at 4 days and to 34.7 mg / kg marc at 6 days. Levels decreased at 10 days to a similar concentration as was present at 0 days. The patterns of change for 4-hydroxy-β-ionone and 3-hydroxy-β-ionone were similar. A five fold increase was observed for 4-hydroxy-β-ionone from 1.0 mg / kg marc at time zero to 6.2 mg / kg marc at four days. A 4 fold increase was observed over the same time period for 3-hydroxy-β-ionone from 1.0 mg / kg marc to 3.8 mg / kg marc. The levels of both compounds decreased markedly after 4 days.

Overall the pattern of change observed for the hydroxy ionones was similar with the compounds reaching a peak during the incubation period followed by a decrease. However, whilst the levels of 4-hydroxy-β-ionone and 3-hydroxy-β-ionone peaked at 4 days the more abundant 3-hydroxy-5,6-dihydro-β-ionone level peaked at day 6. Similarly to the discussion on monoterpenol release in this experiment, possible factors that would explain this observation include variability in the activity of biotransformative enzymes and the rate of conjugate hydrolysis. In relation to the later point, if hydrolysis enzyme activity was rate limited then it is possible that larger pools of specific glycosidic substrates were hydrolysed more slowly. The widespread occurrence of C-13 norisprenoids in plant tissues was extensively reviewed by Winterhalter and Schreier (1994) and the structures of di-oxygenated and higher-oxygenated conjugates were detailed. Only a limited number of hydroxy ionones are observed to be released from marc during this experiment, possibly reflecting the pattern of C-40 carotenoid biosynthesis.

Related patterns of change were observed for the cucurbates (see **figure 6.6**) although the methodology was not able to distinguish between methyl diepicucurbate and methyl epicucurbate. In this group of compounds methyl diepicucurbate showed the highest relative change with levels increasing 9 fold from 0.67 mg / kg marc to 6.13 mg / kg marc at 4 days. Levels of this compound were sustained until 6 days with subsequent decreases at ten days to a level similar to that at the start of the experiment.

The changes in volatile levels of compounds not associated with direct release from glycosidic conjugates allow further discussion with regard to the complexity of processes occurring during the marc incubation. The compounds analysed over the ten day incubation that would not be directly derived through conjugate hydrolysis are 3-oxo-5,6-dihydro-β-ionone (**figure 6.4**), beta-ionone (**figure 6.5**), 7,11 epoxymegastigm-5,6-en-9-one (**figure 6.5**), 7,8-dihydro-β-ionone (**figure 6.5**) and heptadec-8-ene (**figure 6.6**). The increases in these compounds between zero time and day 2 are important to note. These increases ranged from 19% for 3-oxo-5,6-dihydro-β-ionone to 45% for heptadec-8-ene. Each of these compounds then showed different patterns of change during the rest of the experiment.

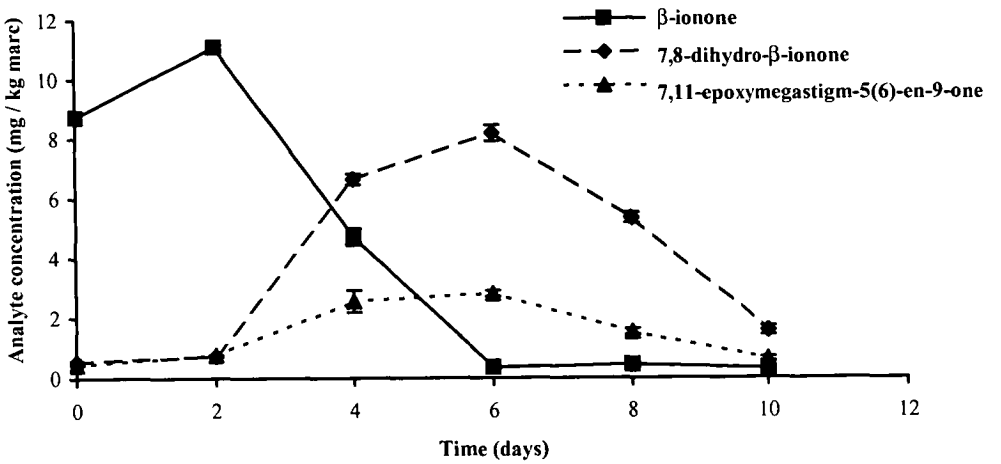


Figure 6.5 Changes in β-ionone, 7,8-dihydro-β-ionone and 7,11epoxymegastim-5(6)-en-9-one levels during a simple ten-day marc incubation trial. Data represents mean and standard errors of 6 replicates.

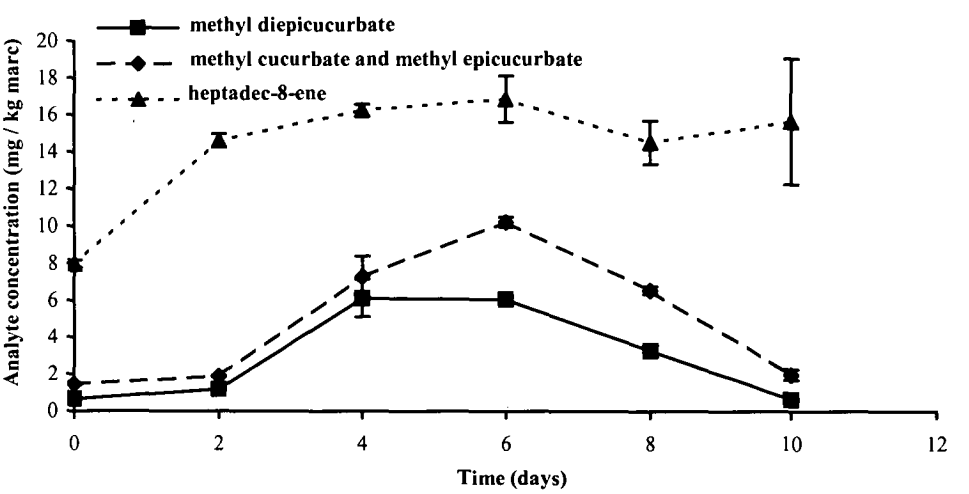


Figure 6.6 Changes in cucurbate and heptadec-8ene levels during a simple ten-day marc incubation trial. Data represents mean and standard errors of 6 replicates.

The increases in these metabolite levels between 0 and 2 days might be explained through release due to breakdown of plant compartments that had previously caused some volatiles to be unavailable to the solvent extraction process. MacTavish (1995) found in preliminary investigations that some forms of tissue disruption of boronia flowers resulted in both increases in extract yield and enhancement of β-ionone concentration in the extract. In this study the marc incubation resulted in a 26% increase in β-ionone between 0 and 2 days. The increases between 0 and 2 days are less likely to be related to the metabolic activity of micro-organisms because temperature profiles indicated that the temperature of the incubation piles did not increase during the first 36 hours of the incubation. This is followed by increases of over 10°C (see **appendix 3**) during the following 12 hours indicating the onset of more metabolic activity in the pile. Further investigation of these factors has the potential to lead to an increased yield of volatiles from the initial extraction of flowers. The development of a commercial process from this marc incubation technique, utilising the increases in volatiles released from conjugates, would not result in increases in the yield of β-ionone due to the disappearance of this compound after 2 days.

The mechanism of changes in the levels of 3-oxo-5,6-dihydro-β-ionone, 7,11-epoxymegastigm-5(6)-en-9-one, and 7,8-dihydro-β-ionone can be considered with regard to possible metabolic processes. These changes may be related to changes in β-ionone and 3-hydroxy-5,6-dihydro-β-ionone levels according to the following rationales. The changes in the levels of 7,8-dihydro-β-ionone and 7,11-epoxymegastigm-5(6)-en-9-one will be considered first. These changes were unexpected as they are not likely to result directly from conjugate hydrolysis. The patterns of change were similar to those observed for several of the monoterpenols and hydroxy C-13 norisoprenoids. 7,8-Dihydro-β-ionone increased from 0.55 mg / kg marc to 8.16 mg / kg marc at 6 days after which the levels decreased and 7,11-epoxymegastigm-5(6)-en-9-one increased from 0.45 mg / kg marc to a maximum level 2.80 mg / kg marc at 6 days followed by a decrease in levels.

The increases in both 7,8-dihydro-β-ionone and 7,11-epoxymegastigm-5(6)-en-9-one match the decrease in β-ionone with regard to timing and actual amounts (mg / kg

marc (**Note:** that the molecular weights of the ionone compounds are roughly similar with differences between mono-oxygenated and di-oxygenated compounds representing less than 10% of the overall molecular weight). In relation to the production of 7,8-dihydro-β-ionone, Hartman *et al.* (1988) observed a similar hydrogenation reaction during the incubation of β-ionone in a microbiological culture.

Sefton and Williams (1991) reported that enzyme preparations derived from fungal sources have led to the accumulation of oxidation artefacts. These artifactual changes included the production of 3-oxo-β-damascone and 3-oxo-α-damascone with a concurrent decrease in 3-hydroxy-β-damascone. Whilst fungal activity during the marc incubation process needs to be explored further, similar activity might result in the oxidation of 3-hydroxy-5,6-dihydro-β-ionone to 3-oxo-5,6-dihydro-β-ionone.

Although this may counter the explanation for the production of 7,8-dihydro-β-ionone and 7,11 epoxymegastigm-5,6-en-9-one from β-ionone another possible mechanism for the increases in 3-oxo-5,6-dihydro-β-ionone includes fungal biotransformation from the β-ionone present in the marc. Related to this Hartman *et al.* (1988) found that incubation of β-ionone in fungal cultures of *Cunninghamella blakesleana* and *Aspergillus niger*, variously resulted in hydroxylation and further oxidation to the oxo derivative in both the 3 and 4 positions of the β ring. This included the production of 4-oxo-7,8-dihydro-β-ionone through incubation with *Cunninghamella blakesleana*. Hydroxylation of β-ionone by *Aspergillus niger* was also observed by Larroche *et al.* (1995). Two possibilities exist then for the generation of 3-oxo-5,6-dihydro-β-ionone during the simple marc incubation process. The first and simplest explanation for the appearance of 3-oxo-5,6-dihydro-β-ionone is through fungal mediated oxidation of the hydroxy analogue. The alternative possibility is through a three-step process of hydroxylation of β-ionone in the three position followed by oxidation and hydrogenation of the double bond in the 5,6 position.

In summary there is possibly a range of processes that occurred during the marc incubation. They include:

1. Release of conjugates through glycosidase action with the possibility that this hydrolysis is rate limited.
2. Simple biotransformative processes including hydrogenation, hydroxylation and oxidation.
3. Extensive biodegrative processes that might include use of the secondary products as an energy source for the microorganisms.
4. The breakdown of compartments that might release volatiles that were previously unavailable to solvent extraction

Whilst this speculation is important more extensive studies with regard to the appearance and disappearance of volatiles would be necessary to develop a more complete picture of the biological processes that occurred during the marc incubation. However, the purpose of this study was to find a mechanism for increasing volatiles through a commercial post-harvest process and the data provided an excellent basis for a commercial trial of the simple marc incubation technique. The data indicated that optimum volatile release occurred in the 4 – 6 day period after which volatile levels began to reduce, presumably due to fungal metabolism. Whilst many of the volatiles reached a maximum level at 6 days, observations with regard to the organoleptic character of the marc indicated that unpleasant phenolic sensations increased during the trial. In addition, the large increase in 3-oxo-5,6-dihydro-β-ionone from 1.6 mg / kg marc at 4 days to 10.6 mg / kg marc at 6 days alluded to the possibility that significant oxidation processes are beginning to impact on the process after this time. For these reasons a 4 day time period was chosen for a commercial scale trial for which the data is presented in the following section.

6.3.5 Commercial Trial

A dark green extract weighing 1.4 kg was obtained from clone 3 marc that had been incubated on a commercial scale. Temperature changes were monitored throughout the incubation period and the data (see **appendix 3**) showed that the temperature decreased over the first 18 hours from 25°C to 16.5°C. The initial temperature was higher than ambient temperatures because warm marc, that had been steamed to remove solvent, was arranged into a pile for incubation immediately following the

first commercial extraction. Temperatures then increased slowly until about 20°C over the next 18 hours which was followed by a relatively rapid increase in temperature from 20°C to 30°C over the next 12 hours. The temperature then began to decrease reaching 27°C after a further 48 hours. The total yield from the initial extraction for this batch of flowers was 3.87 kg and the marc extract yield of 1.4 kg represents a yield addition of 36 %. The percentage of absolute by weight remaining after dewaxing of a 5.0 g test sample was 87%. This compared favourably with the primary flower concrete which usually contains 50% absolute by weight.

Organoleptic Testing of Marc Extract

Gas Chromatography / Olfactometry of the commercial extract revealed that many aroma characteristics that would complement the boronia extract, including spice and fruit characters, eluted on GC after octadecane. The aroma characteristics in this region included spice and fruit characters and the results of a GC / O analysis are presented as an aromagram in **table 6.5**. There are two key aspects to this aromagram. Firstly, no aroma sensations were detected in the region where norisoprenoid and cucurbate compounds eluted. Secondly, many of the aroma sensations were related to compounds that eluted after octadecane. These aroma characteristics were predominated by fruit sensations and also included spice, cinnamon and floral characters. These are characteristics that are compatible with boronia extract but relate to compounds that have not previously been assessed in the quality control analyses of boronia extracts.

Olfactory Dilution Analysis, which indicated that the marc contained tropical fruit aroma characters including persimmon and pomegranate, were supportive of the GC/ O results. The three main groups of volatile compounds in the post octadecane region, previously detected by GC (Weyerstahl *et al.*, 1995), which were considered as possible contributors to the aroma sensations described are the tiglamides, cinnamate esters and 8-hydroxy linalool esters. It is also important to note that GC / O has the potential to detect odour active compounds that are not detected by GC / MS (Friedrich and Acree, 2002).

Time Stop Watch	Time Peak ID	Aroma characteristics	Chromatogram region or peak
7.50	7.31	Sweet vanilla essence	
10.00			
10.47		Green banana	
12.00	12.54	Floral – boronia top note	
13.40		Cucumber	
14.35		Slight woody aroma	
19.50	19.64	Spicy	
21.00		Woody and citrus	
22.10	22.23	Woody	
24.10	24.16	No Aroma	Hydroxy C-13 norisoprenoid
24.40	24.76	No aroma - new peak	region starting here
26.08	26.19	Touch of cinnamon	Heptadec-8ene
	29.65		Octadecane (C-18) - internal standard
31.30		Slight spicy	No peak present
32.25 - 35.00	34.97	Lime	First broad peak after C-18
36.00		Cinnamon / citrus	very small peak
36.5	36.74	Orange / grapefruit	very small peak
37.4		Lime	
38.18	38.22	Floral and sweet (linalool like)	large broad peak
38.55		Lime	
	39.59	Cinnamon / woody / slight lime (floral at base of peak)	
41.2	41.67	Tropical fruit (mango / pawpaw?)	
42.15		Papaya	
42.58	43.16	Grapefruit changing to mandarin and orange	first sharp peak of three
43.5		Hint of cinnamon	
44.3		Baked food	
45.36	45.48	Quince	large sharp peak - largest in post C18 region
48.15	48.42	Fruity? - plus hay	last significant peak

Table 6.5 Aroma sensations of marc compounds detected using GC / Olfactometry.

GC Analysis of Volatiles

Compounds in the marc were identified using GC / MS and analysed by GC / FID. Using mass spectrometric identification, peaks that contained more than one compound were excluded from the FID analysis. **Table 6.6** shows the levels of several key volatile compounds in the marc extract that might have the potential to contribute aroma value to the extract.

Compound	% (w/ w)
Linalool	0.18
7-hydroxy hotrienol	0.20
β-ionone	0.20
7,11- epoxymegastigm-5,6-en-9-one	0.25
3-oxo-5,6-dihydro-β-ionone	0.32
3-hydroxy-5,6-dihydro-β-ionone	1.98
4-hydroxy-β-ionone	0.37
3-hydroxy-β-ionone	0.48
methyl cucurbate	0.17
methyl epicucurbate	0.30
heptadec-8-ene	2.03
total volatiles eluting prior to octadecane	14.52
8-hydroxy-linalyldecanoate	0.70
N-[2-(4-Prenyloxyphenol) ethyl] tiglamide	1.56
methyl (Z)-4-geranyloxy)cinnamate	2.03
methyl (E)-4-(geranyloxy)cinnamate	6.24

Table 6.6 The percentage of volatiles by weight in the marc extract obtained by a commercial-scale process (relative to octadecane assuming a response factor of 1).

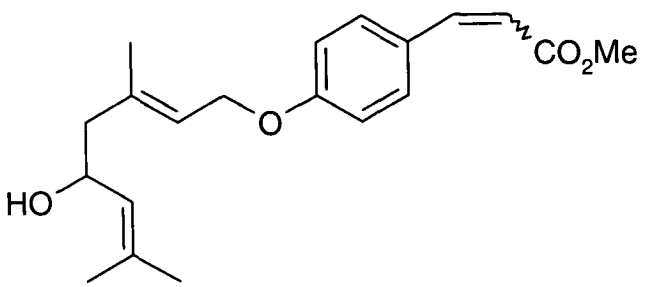
Analysis of the extract indicated the presence of a number of volatiles that might be expected to be released from glycosides as was indicated in the ten day experiment. The percentage by weight of volatiles eluting prior to octadecane was 14.5%. This compared to a percentage of 19.5% for the primary extract from the flowers that were utilised for the marc incubation study reported here. Additionally, this is within the range of 12 – 18% of extract normally obtained for primary extracts from boronia flowers. However it is important to note that the percentage of volatiles by weight in the extract included the most volatile "top notes" α-pinene and β-pinene, which almost certainly were derived as contaminants in the solvent that is routinely recycled without purification as part of the commercial process. This amounted to 2.4% of the total volatiles that elute on GC before octadecane.

Monoterpene volatiles that were observed to increase during the ten day trial and that were present in the marc extract included linalool and 7-hydroxy hotrienol whilst 8-hydroxy linalool was not detected. The C-13 norisoprenoid with the highest level in the marc extract was 3-hydroxy-5,6-dihydro-β-ionone (1.98 % w/w) which is consistent with the data from the ten day trial. Lower levels of 4-hydroxy-β-ionone and 3-hydroxy-β-ionone were present in the marc extract along with the cucurbate compounds and a range of volatiles (β-ionone, 7,11- epoxymegastigm-5,6-en-9-one, 3-oxo-5,6-dihydro-β-ionone, and heptadec-8-ene) that would not have been present as glycosides. The high level of heptadec-8-ene was consistent with the data obtained in the ten day trial and may indicate that this compound was not extracted efficiently in the primary extraction from the flowers. Inconsistent with the ten day trial, 7,8-dihydro-β-ionone was not detected in the marc. The reasons for differences in relative amounts of volatiles, observed between the ten day trial and the commercial trial, may include aeration of the incubating marc with regard to working with a larger volume. Future experiments may be needed to understand the potential for variation in this process.

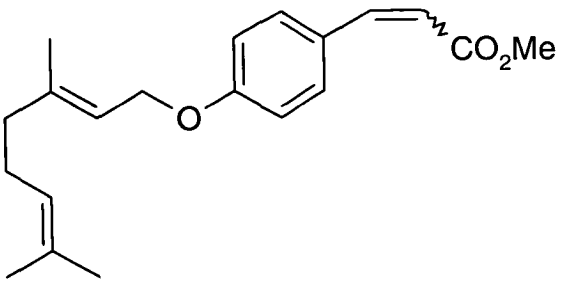
Given that the organoleptic profiling was suggestive that several post C18 eluting compounds contributed to the flavour and aroma of the extract, several of these compounds were also measured. The compounds measured in this analysis included 8-hydroxylinalyl decanoate, N-[2-(4-Prenyloxyphenol) ethyl] tiglamide, and methyl (E)-4-(geranyloxy)cinnamate. The structures of these compounds and structurally related compounds previously detected by Weyerstahl et al. (1995) are presented in **Figure 6.7**. These compounds represent 10.8% by weight of the total extract. Future work that leads to understanding the role of these compounds with regard to the flavour and aroma of the extract will be important. However, all things considered, the commercial trial has demonstrated that an increased yield with favourable organoleptic properties can be obtained from the marc. This process requires further experimentation to maximise the overall yield and organoleptic properties of the marc extract.

Direct Probe Mass Spectrometry

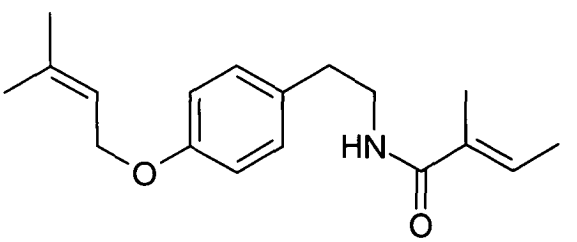
MacTavish (1998a) referred to unpublished work that had provided evidence for the presence of triglycerides and of α- and β-amyrin (triterpenoid alcohols) and their



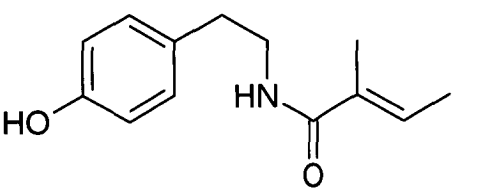
(E) and (Z) isomers of
Methyl-4-(5-hydroxygeranyloxy) cinnamate



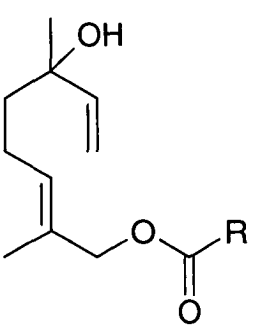
(E) and (Z) isomers of
Methyl-4-(geranyloxy) cinnamate



N-2-[(4-Prenyloxyphenyl) ethyl] tiglamide



N-2-[(4-Hydroxyphenol) ethyl] tiglamide



8-Hydroxy linalyl esters
R = C7 – C13

Figure 6.7 Structures of selected cinnamates, tiglamides and 8-hydroxy linalyl esters that have been detected in boronia flowers.

long chain fatty acid esters. That work, which had been performed using direct probe mass spectrometry (Davies, 2002, personal communication) was re-examined and new analyses were performed that allowed insight into the differences in the less volatile compounds (appearing on GC after octadecane) between the conventional concrete and absolute, and the marc extract obtained in the commercial trial. The earlier work had revealed ions that were characteristic of tiglamides, cinnamates, wax alkanes (C-21 – C-31), tocopherol, the amyrin compounds (described above), and triglycerides. **Table 6.7** lists characteristic ions for each of these compounds.

Compounds	**Characteristic ions**
Tiglamides	120
Cinnamates	178
Wax alkanes (C21 – C33)	57
α- and β-Amyrin	218
α- and β-Amyrin esters	218, 426, 664
Tocopherol (vitamin E)	430
Triglycerides	577

Table 6.7 Characteristic ions for compounds eluting after octadecane on GC that are suitable for distinguishing these compounds in a direct probe mass spectrometry analysis.

A comparative examination of spectral averages (see **appendix 4**) allowed some broad comments to be made with respect to the differences between the samples. Firstly the ratio of cinnamates to the amyrin compounds increased in the absolute compared to the concrete. This indicated that the amyrin compounds are partly removed during absolute production, which involves removal of waxes through solubilisation in ethanol at -20°C. In addition a large increase in the ratio of the cinnamate ion to the amyrin related ion was apparent in the marc extract compared to both the absolute and the concrete. This might be due to efficient extraction of these compounds from the flowers in the first commercial extraction or due to metabolism of these compounds in the marc incubation. Notably the relative amount of

tocopherol increased in the marc sample. The tiglamide ion at *m/z* 120 was apparent in all samples. Additionally, small ion peaks were indicative of small levels of triglycerides, and β-amyrin palmitate in all of the samples.

The relative amounts of the less volatile compounds would be important if they provide stabilisation or act as a "base" for the volatile aroma and flavour compounds. An awareness of these less volatile compounds may also be relevant in considering further extraction of useful compounds from boronia. Related to that, β-amyrin palmitate has been found in several different plant species (Yadagiri *et al.*, 1984; Chaudhuri and Chawla, 1987) and there is evidence that it may have efficacy as a naturally occurring anti-depressant (Subarnas *et al.*, 1993).

Chapter 7

Testing of Fungal Isolates

7.1 Introduction

The possible role of endogenous fungi in the release of volatiles from boronia marc was highlighted in chapter 6 whereby volatiles were observed to increase during a simple incubation of the marc on the factory floor. This chapter was intended to examine that process in further detail through (1) isolation of fungal colonies growing in the marc and (2) testing of the fungal isolates on sterilised marc.

7.2 Materials and Methods

7.2.1 Materials

Boronia megastigma (Nees) clone 3 marc was obtained from Essential Oils of Tasmania following commercial extraction of the flowers. Marc was stored in a freezer (-20°C) prior to sterilisation and experimentation. Pentane and hexane were redistilled prior to use. All other solvents were analytical reagents. Sabouraud Dextrose Agar was obtained from Oxoid.

7.2.2 Isolation of Endogenous Fungi from Marc

Regions of obvious microbiological growth from marc that had been allowed to incubate at room temperature for 4 days were sampled and plated onto: 1. Basal Mineral Salts containing 1.5% agar and 2. Basal Mineral salts containing 1.5% agar plus 0.05% boronia glycosidic extract (see chapter 4). Nine isolates from plates which had been incubated at 25°C were selected on the basis of either comparative growth on the glycosidic extract and / or by organoleptic properties of a newly

opened plate that contained the boronia glycosidic extract. Each isolate was tested in the marc incubation system (**section 7.2.4**) for an ability to cleave glycosides in a preliminary trial. For the preliminary trials a small piece of gel infected with the fungi was excised from Sabauroud plates and placed in a sterilised jar containing a small amount of sterilised water. This was shaken and the water and gel were poured over the marc which had been previously weighed into sterilised containers. A further four isolates (9, 7a, 2a and 8) were then selected for quantitative incubation studies.

Recipes for Microbiological Media

Basal Mineral Salts

5.0 g/L NH_4NO_3

2.0 g/L K_2HPO_4

0.2 g/L $MgSO_4.7H_2O$

0.01 g/L $CaCl_2.2H_2O$

0.01 g/L $FeCl_3.6H_2O$

Sabouraud Agar

65 g/L Sabouraud Dextrose Agar

Solutions were sterilised by autoclaving at 100 kPa for 20 minutes

7.2.3 Identification of Fungi

Fungi were identified to genus level on the basis of the morphological features of the hyphae and sporangiophores. Fungi 7a and 9 were identified as *Penicilium* species whilst fungi 2a and 8 were identified as *Aspergillus* species (Personal communication, Dr Martin Line, 2005).

7.2.4 Marc Incubation

Approximately 30 g of boronia marc which had been sterilised by irradiation (Steritech, Melbourne) was transferred to a sterile pottle and inoculated with a designated fungal isolate. Inoculation was achieved by excising a 1 cm diameter piece of gel infected with the fungi from growth plates (Sabauroud, 25°C) and placing it into the pottle containing the sterilised marc. Samples were then incubated

in a constant temperature oven at 25°C for up to 4 days depending on the experimental protocol.

Protocol 1: *Quantitative Fungal Trial – Test samples were incubated for four days.* The time period of four days was selected because it was the time selected for the commercial trial using the simple marc incubation process described in chapter 6.
Protocol 2: *Time Trial – Test Samples were incubated for 1, 2, 3 or 4 days.*
Results from Protocol 1 indicated the need for a more extensive time trial with selected fungal isolates (fungi 2a and 7a, see results section).

7.2.5 Sample Extraction

Samples were transferred to a conical flask and extracted with petroleum ether according to the method of MacTavish and Menary. (1999a). Accordingly, the incubated marc samples were extracted three times with pentane : hexane (80:20) by first washing for 120 min followed by two subsequent washes for 60 min each. Samples were given a final rinse in 50 mL of solvent. Each of the samples containing solvent was shaken for the extraction period using a Gallenkamp Cooled Orbital Shaker at 100 rev / min at room temperature. After each extraction the solvent extracts were filtered through cotton wool, combined, and stored at 4°C prior to solvent removal with a rotary evaporator. Each sample was rotary evaporated to dryness at 35°C and then subjected to a final rotary evaporation drying period at 50°C for 5 min.

7.2.6 Preparation of Samples for GC / MS Analysis

An internal standard (octadecane, 50 μL = 101.1 μg) was added to the evaporated samples in a 250 mL round bottom flask. The samples were then redissolved in 4 mL of dichloromethane. A portion of each sample was transferred to GC sampling vials for analysis and the remainder stored in glass vials for further aroma profiling after evaporation of the solvent.

7.2.7 Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

Petroleum ether extracts were analysed using a Varian CP-3800 gas chromatograph equipped with a 25 m x 0.32 mm x 0.52 μm film thickness Hewlett Packard HP1 column. Injections were made with a Varian 1177 injector at a temperature of 230°C in a split mode (30:1). The carrier gas was helium at a flow rate of 2.5 mL / min. The initial oven temperature was 60°C with increases at 12°C / min to 120°C, then 6°C / min to 230°C, and 20°C / min to 290°C. Sample detection was performed with a Varian 1200 triple quadropole mass spectrometer with scanning over the *m/z* range 35 to 420 in electron ionisation mode. Settings were ion source temperature 200°C and transfer line temperature 280°C.

Analysis was performed using a Varian Mass Spectrometry Workstation Version 6.30. Peaks were identified by a combination of commercial (NIST, Version 2.0) and in-house libraries. Mass chromatograms of diagnostic ions for analytes were extracted from the data and the results are expressed as a ratio to the internal standard (octadecane).

7.2.8 HPLC / MS Analysis

Methanol extracts of boronia were analysed for changes in glycosidic conjugates using HPLC / MS. Analysis was performed with a Waters 2690 HPLC and Finnigan LCQ. The HPLC was equipped with a Waters Novapak C18 3.9 mm x 150 mm column and an Alltech Econosphere C18 guard cartridge.

HPLC Program

The mobile phases were A. methanol (100%) and B. 0.1 M ammonium acetate (pH 6.38). Initial conditions were 30% A / 70% B with a linear gradient to 70% A / 30% B at 25 minutes. The flow rate was 0.8 mL / min with an injection volume of 5 μL.

Mass Spectrometry

MS conditions were APCI source. APCI Vapouriser Temperature: 460°C; Capillary Temperature: 170°C; Sheaf Gas Flow: 60 psi; Capillary Voltage: 41 V. Data

collection for the malonyl glycosides of C-13 norisoprenoids was from the mass chromatograms at *m/z* 457 and 459 from specific MS^2 experiments. Similarly the data for malonyl glycosides of cucurbates was derived from MS^2 experiments targeting ions at *m/z* 209, 227, 475. Data for the other glycoside grouping was derived from $[M+NH_4]^+$ ions in normal scan MS experiments. Full Scan analysis was over the range *m/z* 150 – 750 at 1 cycle / s. Finnigan Navigator software was used to generate and analyse the data.

7.2.9 Olfactory Testing of Fungal Products from the Fungal Trials

Experimental samples from laboratory scale tests were examined with regard to their aroma profile at various stages during the extraction process. A preliminary organoleptic assessment was done at several stages during experiments including by

(1) sniffing of samples prior to extraction

(2) olfactory dilution analysis

(3) use of tapers.

These testing procedures were used as a factor in selecting fungal isolates, from the initial four day experiment, for further experimentation. For the **dilution analysis** a 1% solution (up to 6 drops) of the extract in ethanol was added to 100 mL of water in a wine glass. This aqueous dilution was then swirled and sniffed. The dilution analysis of the laboratory scale extracts from the four day experiment was completed in two stages. Initially, one sample from each experimental group was tested by initially adding 2 drops of the 1% solution. The aroma profiles were determined again after twice adding a further two drops. Following this a more thorough investigation of the aroma profile of each sample was determined by adding 4 drops of the 1% solution. For the analysis with **tapers** the ethanolic samples were tested by dipping a taper into the solution and sniffing the taper during dry out. Testing was performed by one expert assessor with two decades of experience in the development of the Tasmanian boronia industry.

7.3 Results and Discussion

7.3.1 Quantitative Fungal Trial

Analysis of Volatiles

Boronia marc that had been incubated with four different fungal isolates for fours days was extracted and analysed using GC / MS. Changes to volatile levels following this incubation of boronia marc with the four different fungal isolates were determined by single ion monitoring on GC / MS and are presented in **Table 7.1**. The changes in volatile levels are presented as a ratio to the C18 standard allowing changes in the level of each volatile to be quantitatively compared within the different fungal treatment groups. However, the levels of each analyte relative to another compound are not comparable by this method.

The compounds measured in this study that contain alcohol functional groups and would therefore be able to form glycosidic conjugates were examined for increases compared to the control (no fungal treatment). Many of the compounds that increased after the four day incubation, due to one or more fungal treatments, corresponded to previously identified glycosidic conjugates. These compounds belonged to the following categories:

1. Monoterpene alcohols

Linalool and 7-hydroxy hotrienol increased due to all fungal treatments with the highest increases observed for the treatments with fungus 7a and fungus 9. An unknown linalool related compound increased in all treatments except with fungus 9. Additionally, 8-hydroxy linalool decreased during incubations with all four fungal isolates.

2. C-13 norisoprenoids

The changes to the hydroxy ionones varied between fungal treatments. The highest relative increases were observed for 3-hydroxy-5,6-dihydro-β-ionone following treatment with fungus 2a and fungus 8. Treatment of marc with those fungi resulted in increases in 3-hydroxy-β-ionone and corresponding decreases in 4-hydroxy-β-

Component	Control	Fungus 9	Fungus 7a	Fungus 2a	Fungus 8
Phenolic and related compounds					
2-phenylethanol	0.5 ± 0.2	0.0	0.0	4.5 ± 0.2	5.7 ± 1.3
4-vinylphenol	0.0	0.0	0.0	0.0	0.0
Monoterpene alcohols					
Linalool	1.1 ± 0.2	10.1 ± 2.7	14.8 ± 1.7	6.5 ± 0.3	6.5 ± 0.9
pinocarveol *	2.3 ± 0.6	3.7 ± 1.2	3.5 ± 0.4	9.2 ± 0.7	4.9 ± 0.5
verbenol *	1.3 ± 0.1	1.6 ± 0.4	2.5 ± 0.3	3.0 ± 0.1	2.6 ± 0.4
borneol *	trace	1.6 ± 0.3	1.7 ± 0.1	12.8 ± 0.7	9.4 ± 0.8
7-hydroxy-hotrienol	18.9 ± 0.8	77.0 ± 5.2	80.0 ± 2.7	61.2 ± 2.0	60.2 ± 4.8
unknown linalool related	2.7 ± 0.3	trace	110.3 ± 5.6	97.8 ± 10.2	160.2 ± 5.5
8-hydroxy-linalool	63.3 ± 2.3	0.0	1.3 ± 0.2	8.6 ± 1.6	19.2 ± 2.4
Other boronia compounds					
β-caryophyllene	2.8 ± 0.5	1.6 ± 0.4	3.1 ± 0.2	3.3 ± 0.1	2.6 ± 0.5
Dodecanol	7.3 ± 0.4	0.0	0.0	0.0	0.0
Spathulenol	4.1 ± 0.3	3.3 ± 0.5	3.3 ± 0.5	3.2 ± 0.2	3.0 ± 0.3
dodecyl acetate	7.5 ± 0.5	0.0	0.0	0.0	0.0
heptadec-8ene	22.2 ± 1.5	5.3 ± 1.4	12.7 ± 1.3	18.1 ± 1.8	15.7 ± 3.4
C-13 Norisoprenoids					
β-ionone	52.1 ± 2.7	31.5 ± 2.6	25.6 ± 2.1	0.8 ± 0.4	0.1 ± 0.1
dihydroactinidiolide	5.2 ± 0.5	0.0	0.0	3.3 ± 0.1	3.9 ± 0.2
Unknown (MW 194)	1.1 ± 0.1	0.0	trace	0.5 ± 0.1	0.5 ± 0.1
7,11-epoxymegastigm-5(6)-en-9-one	4.6 ± 0.2	10.8 ± 1.0	3.9 ± 0.8	3.4 ± 0.6	3.1 ± 0.3
Unknown (MW 210)	0.0	18.5 ± 3.3	56.1 ± 3.9	13.5 ± 1.0	16.7 ± 1.1
3-oxo-5,6-dihydro-β-ionone	4.1 ± 0.3	237.3 ± 9.4	161.4 ± 3.3	38.8 ± 2.4	51.0 ± 2.7
Unknown (MW 210)	1.8 ± 0.3	9.4 ± 0.5	13.4 ± 0.2	14.9 ± 0.2	13.2 ± 0.4
3-hydroxy-5,6-dihydro-β-ionone	39.3 ± 1.4	72.4 ± 1.5	51.8 ± 2.1	117.3 ± 2.8	118.8 ± 2.0
?? 3-oxo-β-ionone	0.2 ± 0.1	1.2 ± <0.1	0.1 ± 0.1	1.9 ± 0.1	4.1 ± 0.1
4-hydroxy-β-ionone	11.2 ± 0.5	15.5 ± 0.8	11.3 ± 0.5	4.4 ± 0.1	6.4 ± 0.5
3-hydroxy-β-ionone	21.1 ± 0.6	19.5 ± 1.2	4.2 ± 0.5	42.5 ± 1.2	47.5 ± 1.5
Unknown (MW 210)	trace	18.5 ± 2.0	20.9 ± 1.6	38.5 ± 0.8	30.9 ± 1.3
Cucurbates					
Methyl diepicucurbate	3.4 ± 0.3	6.8 ± 0.3	14.9 ± 1.0	1.5 ± 0.1	1.4 ± 0.2
Methyl cucurbate	2.5 ± 1.7	2.3 ± 0.2	3.3 ± 0.3	1.9 ± 1.0	1.1 ± 0.1
Methyl epicucurbate	2.5 ± 0.2	6.9 ± 0.3	9.5 ± 0.4	1.6 ± <0.1	1.9 ± 0.1
8-Hydroxy-linalyl esters					
8-hydroxylinalyl octanoate	44.0 ±	0.0	0.0	0.0	0.0
8-hydroxylinalyl nonanoate	36.7 ±	0.0	0.0	0.0	0.0
8-hydroxylinalyl decanoate	78.0 ± 5.1	0.0	3.7 ± 1.0	0.6 ± 0.3	0.0
8-hydroxylinalyl undecanoate	5.9 ± 0.6	0.0	0.3 ± 0.3	0.0	0.0
Tiglamides					
N-[2-(4-prenyloxyphenyl) ethyl] tiglamide	539.3 ± 28.4	130.1 ± 13.9	498.6 ± 31.7	42.9 ± 9.5	72.9 ± 10.0
unknown (*m/z* 120, 204)	0.0	189.4 ± 14.6	0.0	36.2 ± 2.3	22.9 ± 1.1
unknown (*m/z* 120, 99, 218)	24.3 ± 3.5	12.1 ± 0.2	17.6 ± 1.5	20.5 ± 1.4	23.6 ± 0.8
Cinnamates					
Methyl (Z)-4-(geranyloxy)cinnamate	81.2 ± 4.0	7.9 ± 2.5	49.7 ± 5.2	73.4 ± 5.2	75.5 ± 11.1
Methyl (E)-4-(geranyloxy)cinnamate	73.8 ± 3.2	15.4 ± 3.4	63.2 ± 7.5	63.4 ± 5.3	67.2 ± 8.8
Methyl (Z)-4-(5-hydroxy-geranyloxy)cinnamate	189.3 ± 5.9	2.4 ± 0.6	34.5 ± 1.9	61.1 ± 6.1	60.7 ± 5.5
Methyl (E)-4-(5-hydroxy-geranyloxy)cinnamate	431.6 ±13.7	5.3 ± 1.5	44.8 ± 5.8	88.7 ± 8.9	125.1 ±10.7

Table 7.1 Comparative effect of fungal isolates on the components of an extract from boronia marc following a four day incubation. Analytes marked with an asterisk were not previously identified by Weyerstahl *et al.* (1995). The results are a ratio of the area of diagnostic ions to the internal standard.

ionone. The treatments with fungus 9 and fungus 7a resulted in variable results for all three analytes.

3. Cucurbates

Treatment with fungus 7a for four days resulted in increases in all three cucurbate compounds (methyl 2,3-di-*epi*-cucurbate, methyl cucurbate and methyl 3-*epi*-cucurbate). Fungus 9 was the only other treatment that led to increases in cucurbates (methyl 2,3-di-*epi*-cucurbate and methyl 3-*epi*-cucurbate). The other fungal treatments led to decreases in the cucurbate compounds.

Other hydroxy compounds that increased due to fungal treatments included 2-phenylethanol, pinocarveol, verbenol, and borneol. 2-phenylethanol is an important aroma compound which imparts a rose-like floral character to extracts (Latrasse, 1991). This compound was found to increase after treatment with fungus 2a and fungus 8. The other two treatments resulted in the disappearance of this compound. The three bicyclic monoterpenes (pinocarveol, verbenol, and borneol) which increased during fungal treatments had not previously been identified in boronia (Weyerstahl *et al.*, 1995). Two alcohols previously identified in boronia, dodecanol and spathulenol, were also analysed during this experiment. These compounds either decreased or remained at similar levels during all fungal treatments.

The bicyclic monoterpenes were found in the control incubations indicating the possibility that they are naturally present in boronia and that increases may be due to conjugate hydrolysis. Equally they may be generated through fungal activity from precursor monoterpenes. Demyttenaere (2001) has published an extensive review on the biotransformation of terpenoids. The author details biotransformations by various micro-organisms to these compounds from α-pinene and β-pinene which are both normally found in boronia extracts. However there is no reason to believe that the later mentioned compounds are available in the marc for such biotransformations as they are very non-polar compounds that should be easily extracted during the commercial extraction process with petroleum ether. Direct biosynthesis by the fungi from carbohydrate sources might also be considered.

The results also indicated that many of the compounds measured in this study and normally found in boronia either decreased or remained unchanged following a four day incubation with each fungus. These compounds included β-caryophyllene, dodecyl acetate, heptadec-8-ene, β-ionone, dihydroactinidiolide, 8-hydroxylinalyl octanoate, 8-hydroxylinalyl nonanoate, 8-hydroxylinalyl decanoate, 8-hydroxylinalyl undecanoate, N-[2-(4-prenyloxyphenyl)ethyl] tiglamide, methyl (Z)-4-(geranyloxy)cinnamate, methyl (E)-4-(geranyloxy)cinnamate, methyl (Z)-4-(geranyloxy)cinnamate, and methyl (E)-4-(5-hydroxygeranyloxy)cinnamate.

The β-ionone which had remained in marc after the primary commercial flower extraction, decreased following all fungal treatments. Whilst treatment with fungi 2a and 8 resulted in almost complete elimination of this compound, only a partial effect was observed for fungus 9 and fungus 7a. Increases in some unknown C-13 norisoprenoids and some oxo forms of the ionones (eg. 3-oxo-5,6-dihydro-β-ionone) were also observed.

The 8-hydroxylinalyl esters, tiglamides, and geranyloxy cinnamates are important to consider because the organoleptic observations, relating to the marc extract discussed in chapter 6, indicated these compounds have the potential to contribute positively to the organoleptic profile of extracts. The 8-hydroxy linalyl esters examined here responded similarly to each fungal treatment. After 4 days the levels of these compounds were either completely undetectable or reduced to relatively minimal levels. The cinnamates were also reduced by most fungal treatments although some variability in the response was observed. All of the fungal treatments resulted in large reductions in both of the 5-hydroxy-geranyloxy cinnamates with fungus 9 and 7a appearing to be more effective in reducing the levels of these compounds. Fungi 8 and 2a did not appear to impact the levels of the two geranyloxy cinnamates whilst fungus 9 clearly had more effect than fungus 7a.

Large changes in the level of N-[2-(4-prenyloxyphenyl)ethyl] tiglamide were observed for all fungal treatments except fungus 7a. It was interesting to further note that the increases in the levels of a tentatively identified tiglamide (*m/z* 120, 204) from a control value of zero were observed for all treatments except fungus 7a where

it remained undetected. A small decrease in the level of the other tentatively assigned tiglamide (*m/z* 99, 120, 218) was observed following treatment with fungus 9.

Analysis of Conjugates

The data presented in **Figure 7.2** showed the effect of the four day fungal incubations on the conjugate classes previously identified through HPLC / MS screening (**chapter 4**) and enzymatic hydrolysis (**chapter 5**). The data measurement process was unable to determine changes to the malonyl glycosides of monoterpenediols due to interference caused through the treatments. Therefore the following comments do not refer to that conjugate group.

Components	control	Isolate 9	Isolate 7a	Isolate 2a	Isolate 8
Malonyl glycosides of C-13 norisoprenoids	11.6 ± 0.8	t	3.3 ± 0.2	8.7 ± 0.4	8.7 ± 0.1
Simple glycosides of C-13 norisoprenoids	79.8 ± 2.9	nd	t / nd	t	t
Malonyl glycosides of cucurbates	3.5 ± 0.4	t / nd	t / nd	2.1 ± 0.1	2.0 ± 0.1
Malonyl glycosides of monoterpenediols	43.2 ± 0.5	nd	Nd	6.7 ± 0.5	6.7 ± 0.6
Simple glycosides of monoterpenediols	16.1 ± 1.9	nd	t / nd	t	†
Malonyl glycosides of monoterpenediols	13.3 ± 1.0	‡	‡	‡	‡

Table 7.2 Effect of different fungal isolates on conjugates in boronia marc. The results are expressed as means and standard errors of four replicates.
† - Not measurable due to interference with rutin. However, they are likely to be considerably reduced.
‡ - Not measurable due to interference from peaks arising due to the fungal treatments.
t = trace. nd = not detected.

Treatment with isolate 9 was demonstrated to result in almost complete hydrolysis of all measurable glycoside groupings. Isolate 7a had a similar impact except that the malonyl glycosides of C-13 norisoprenoids were not completely hydrolysed. Treatments with isolates 2a and 8 resulted in similar hydrolysis patterns that were substantially different to the other isolates. Both of these isolate treatments resulted

in disappearance of the simple glycosides of C-13 norisoprenoids and monoterpenols. However, these isolates had a more limited capacity to result in hydrolysis of the malonyl glycosides of C-13 norisoprenoids, monoterpenes and cucurbates. The malonyl glycosides of monoterpenediols were reduced by 85% whilst the malonyl glycosides of C-13 norisoprenoids were reduced by only 25%. Overall, it is clear that fungi isolated from boronia have the capacity to variably effect hydrolysis of the glycosidic conjugates isolated from boronia marc.

Organoleptic Assessment

Samples were examined for their organoleptic characteristics by an expert assessor following the four day incubation period and this information was used, along with the quantitative analysis of volatiles, to make decisions regarding further experimentation. The organoleptic assessments were made at several stages during processing of the samples using a variety of mechanisms. These assessments are summarised below:

1. Sniffing of the treated marc contained in pottles immediately following completion of the incubation.
2. Sniffing of the petroleum ether extract contained in dry-down flasks prior to analysis.
3. Dilution analysis of the sample of extract.
4. Sniffing of tapers during dry-out.

The raw data obtained in these organoleptic assessments, which is available in its entirety in **appendix 5**, was summarised for comparative purposes. The protocol used for the dilution analysis component of this summary was based on a preliminary aroma dilution analysis of representative samples from each of the experimental groups and aroma elements in the extract varied according to the dilution. The results of the preliminary analysis (**Table 7.3**) showed that citrus elements were strongest at the lowest dilutions in the control and experimental groups. Tropical fruit and floral elements were present varyingly with floral elements increasing as the dilution was lessened. On the basis of this preliminary analysis a more comprehensive assessment of each sample using 4 drops in 100 mL was then completed.

Experimental Group	2 drops	4 drops	6 drops
Control	Citrus	Citrus	At this dilution all samples gave a similar profile to that obtained with less drops, with floral characteristics increasing in each of the treatments as drops were added.
Fungus 9	Citrus	Citrus plus tropical fruits. Slight Apricot.	
Fungus 7a	Citrus with some tropical fruit.	Citrus and tropical fruit.	
Fungus 2a	Citrus, floral and some tropical fruit.	Tropical fruit, a little citrus, slightly floral.	
Fungus 8	Citrus and tropical fruit.	Floral and fruity background.	

Table 7.3 The effects of different dilutions on the aroma profile of representative samples of each experimental group from the four day experiment.

The summary of all aroma testing, presented in **table 7.4**, was based on recording the number of references by the expert assessor to aroma characteristics that might make either a favourable or negative contribution to the boronia aroma profile. Details of the favourable comments are listed individually in the summary whereas negative responses are pooled and listed together. Each reference was divided into two categories, primary impact (P) and background (B).

All treatments including the control contained positive aroma characteristics. The floral and boronia characteristics were considered to be highly advantageous. Tropical fruit and "other fruit" characteristics were considered to have a higher value than the citrus character. The hay, herbaceous, spice, cinnamon, tobacco and resin characteristics were also considered to give a positive impact.

Overall, treatments with fungus 2a and fungus 8 were considered to impart the most favourable aroma outcomes to extracts when compared to the other treatments. Treatment with both of these fungal isolates most consistently showed floral aroma characteristics across the range of assessment protocols. Importantly, these fungal

treatments resulted in the appearance of floral characteristics at lower dilutions in the preliminary dilution trial (see **figure 7.3**) than was observed for the other treatments. Additionally, all four replicates in the treatment with fungus 2a consistently showed floral characteristics at each assessment stage except in the dry down flasks where tropical fruit and spice were more apparent.

Treatment Group	Aroma character	Pottles		Dry down Flasks		Dilution analysis		Tapers	
	Intensity	P	B	P	B	P	B	P	B
Control	Floral			2	1			1	3
	Boronia			2					
	Citrus	4				3			1
	Tropical fruit						3	3	
	Other fruit			1	1	1			
	Hay			2	2		1	4	
	Herbaceous								
	Spice						1		
	Cinnamon	3							
	Tobacco	4		1	1			1	
	Resin								
	Other negative			1					
	Other positive								1
fungus 9	Floral						1	2	
	Boronia	2							
	Citrus						2	3	
	Tropical fruit							1	
	Other fruit			1		3	1		
	Hay							1	
	Herbaceous								
	Spice			1		3			
	Cinnamon	2							
	Tobacco	3					1		
	Resin								
	Other negative	1	2	3					
	Other positive			2					
fungus 7a	Floral					2		3	
	Boronia		1						
	Citrus	2					1	2	
	Tropical fruit					1			
	Other fruit				4	2	1		
	Hay			2	1				

	Herbaceous				1								
	Spice	1						1					
	Cinnamon	2											
	Tobacco	2	1										
	Resin												
	Other negative		1		3	1		1					
	Other positive		1										
fungus 2a	Floral	4						2	2		3	1	
	Boronia												
	Citrus	3	1										
	Tropical fruit				2	1							
	Other fruit							2					
	Hay											1	
	Herbaceous							1					
	Spice												
	Cinnamon	1	1		2	1			1				
	Tobacco		1		1	1							
	Resinous	2											
	Other negative										1		
	Other positive												
fungus 8	Floral	2	2		2	2		2	1			2	
	Boronia							2					
	Citrus	3			1	1			1				
	Tropical fruit				1	1							
	Other fruit				1	1			1				
	Hay				1	1			1				
	Herbaceous				1	1							
	Spice										1		
	Cinnamon	1									2		
	Tobacco	1											
	Resin	3											
	Other negative	1	2								1		
	Other positive								1			1	

Table 7.4 Summary of observed aroma characteristics of boronia marc and extracts following a four day incubation with fungal isolates. The *Other negative* characteristics were: off odour sulphur; mouldy smell; almost acidic; musky, dusty shoe polish smell; sulphur of cowyard smell, strong shoe polish, fermented, and fermenting brewery smell. The *Other positive* characteristics were: tea, green and herby, hoppy and musk.

The negative characteristics observed in the pottles and dry down flasks were not as strongly apparent in the taper and dilution analyses. Different aroma threshold concentrations for compounds imparting these characteristics may explain this observation in the case of the dilution analysis. Changes due to evaporation of top notes during dry out of the tapers may have also been a factor. The dilution analysis and taper profiling were considered to have the highest priority when considering the aroma characteristics resulting from the different treatments.

Compounds found to be released during this experiment that might be responsible for the floral elements detected in these assessments include 2-phenylethanol, various monoterpenes and ionones. With regard to that, literature references which comment on the aroma characteristics of many of these compounds are detailed in the literature review (**table 1.5**). The monoterpenol, linalool, imparts floral characteristics whilst the monoterpenediol, 8-hydroxy linalool imparts a more complex aroma with woody and herbaceous elements. A woody characteristic in linalool has previously been associated with a floral element (Arctander, 1960b). In addition, several ionone compounds have been described as having a floral characteristic. Citrus elements are common to several hydrocarbon monoterpenes.

Summary

The analytical results from the four fungal treatments allowed the fungi to be divided into two groups showing close alignment in terms of analytical outcomes. The alignments were fungus 9 with fungus 7a and, fungus 8 with fungus 2a. These groupings were supported by identification of the fungal isolates to genus level. Both fungi 7a and 9 were *Penicillium* species whilst fungi 2a and 8 were identified as being *Aspergillus* species.

Whilst an increase in yield of volatiles was observed in this experiment consideration was given to the possibility that the marc had been over metabolised during the incubation period. A number of factors supported this rationale including:

1. Increases in 3-oxo-5,6-dihydro-β-ionone occurred during the incubations. This varied from a ten fold increase through treatment with fungus 2a to a 90 fold increase following treatment with fungus 9. The possibility existed that fungal

enzymes were oxidising the newly formed 3-hydroxy-5,6-dihydro-β-ionone. This rationale was supported by the reverse patterns of increase in these two compounds by the two fungal groups.

2. Large reductions in some groups of compounds. The variable disappearance of 8-hydroxy linalyl esters, tiglamides and cinnamates due to all four fungal treatments was suggestive that unwanted metabolism was occurring over the four day incubation period.

3. No increase in some monoterpene and C-13 norisoprenoid volatile levels. The results indicated that 8-hydroxy linalool decreased during the incubation period whereas it would have been reasonable to expect an increase in this compound similarly to the increases observed for linalool and 7-hydroxy hotrienol. Decreases in 3-hydroxy-β-ionone and 4-hydroxy-β-ionone were also observed following some treatments.

On the basis of the above considerations fungi 7a and 2a were selected for further study. In addition to understanding changes over the four day period due to release from conjugates, the selection of fungi from the two groupings might allow an insight into more complex patterns of glycoside release and further metabolism. These patterns might include the oxidation of released volatiles, biosynthesis of volatile compounds by the fungi, and release of compounds from sources not related to glycosidic precursor breakdown.

7.3.2 Time Trial Experiment

Ionones

The observed increases in ionones are complex and allude to the possibility that several factors are operating due to the fungal treatments. The data representing the effect of both fungal isolates on C-13 norisoprenoids during a four day incubation are presented in **table 7.5**. The data includes a t-test distribution for each analyte. The results are arbitrary units which represent the ratio of the peak areas for diagnostic ions to the peak area of the internal standard. The positively identified ionones which could be derived directly from the breakdown of glycosides (3-hydroxy-5,6-dihydro-β-ionone, 3-hydroxy-β-ionone and 4-hydroxy-β-ionone) are considered first and a graphical representation of the changes are represented in **figure 7.1**.

3-Hydroxy-5,6-dihydro-β-ionone and 3-hydroxy-β-ionone showed similar patterns of change. The control treatment showed small but significant relative increases over the four day incubation period. Treatment with fungus 2a gave the highest increases for both analytes. The fungus 2a treatment resulted in 3-hydroxy-5,6-dihydro-β-ionone increasing from 38 to 132 units after 1 day with no significant change after that time. Treatment with fungus 7a resulted in a smaller but significant increase after 2 days from 38 to 84 units. This was followed by a significant reduction between 2 and 3 days.

3-Hydroxy-β-ionone increased during treatment with fungus 2a from a blank of 9 to 47 units after 1 day with a further smaller but significant change after 3 days. The fungus 7a treatment results for this analyte showed a similar rate of increase up to 2 days followed by a significant reduction in the levels of that analyte after 3 days with no further significant change. The results for 4-hydroxy-β-ionone showed a different pattern to the other "hydroxy" analytes. Treatment with fungus 2a resulted in a significant increase from 8 to 24 units after 1 day with values falling to 11 units after 4 days. This latter value was not significantly different to the blank value. Treatment with fungus 7a resulted in a large increase from 8 to 58 units after 2 days with values then falling significantly on each subsequent day.

	Blank	Control				Fungus 2a				Fungus 7a			
Time (days)	0	1	2	3	4	1	2	3	4	1	2	3	4
Compound													
β-Ionone	70.6^{c} ± 3.2	81.6^{b} ± 3.3	$85.8^{a,b}$ ± 2.4	91.8^{a} ± 2.5	69.1^{c} ± 4.1	59.8^{d} ± 1.8	14.1^{g} ± 2.4	5.0^{h} ± 1.6	1.6^{h} ± 0.9	68.3^{c} ± 3.9	41.1^{e} ± 2.5	29.8^{f} ± 1.4	26.3^{f} ± 1.2
Dihydroactinidiolide	$9.4^{b,c}$ ± 1.1	$11.1^{a,b}$ ± 0.5	$11.3^{a,b}$ ± 0.4	11.7^{a} ± 0.7	$10.3^{a,b,c}$ ± 0.6	$10.9^{a,b,c}$ ± 0.3	$9.3^{b,c}$ ± 0.4	$10.3^{a,b,c}$ ± 0.7	9.0^{c} ± 0.7	$10.3^{a,b,c}$ ± 0.7	1.5^{d} ± <0.1	1.1^{d} ± <0.1	1.0^{d} ± 0.1
Unknown (MW = 194)	1.8^{f} ± 0.3	$2.5^{d,e,f}$ ± 0.4	$3.2^{c,d,e}$ ± 0.2	$3.7^{c,d}$ ± 0.3	3.8^{c} ± 0.2	$2.2^{e,f}$ ± 0.2	1.9^{f} ± 0.2	$2.5^{e,f}$ ± 0.3	$2.5^{d,e,f}$ ± 0.3	6.3^{a} ± 0.5	5^{b} ± 0.9	$2.1^{e,f}$ ± 0.7	2.0^{f} ± 0.2
7,11-Epoxymegastigm-5(6)-en-9-one	2.6^{f} ± 0.4	$5.3^{e,f}$ ± 0.2	$9.4^{d,e}$ ± 1.0	$10.2^{c,d}$ ± 0.4	$10.6^{c,d}$ ± 0.2	$13.8^{b,c}$ ± 1.4	$17.6^{a,b}$ ± 1.0	$15.7^{a,b}$ ± 3.0	$11.4^{c,d}$ ± 1.1	$10.4^{c,d}$ ± 1.6	19.0^{a} ± 2.9	$10.8^{c,d}$ ± 1.5	$8.3^{d,e}$ ± 0.6
Unknown (MW = 210) - 1	0.0^{f} ± 0.0	0.0^{f} ± 0.0	$1.2^{e,f}$ ± 1.2	0.0^{f} ± 0.0	0.0^{f} ± 0.0	$4.6^{e,f}$ ± 0.5	14.8^{d} ± 1.1	25.4^{c} ± 1.1	27.4^{c} ± 2.3	$9.7^{d,e}$ ± 1.3	57.4^{b} ± 4.6	92.5^{a} ± 6.7	91.4^{a} ± 5.0
3-Oxo-5,6-dihydro-β-ionone	6.0^{e} ± 0.6	6.2^{e} ± 0.3	6.7^{e} ± 0.1	7.2^{e} ± 0.1	5.8^{e} ± 0.8	9.9^{e} ± 0.5	30.8^{d} ± 1.0	44.8^{d} ± 1.9	43.5^{d} ± 1.6	33.0^{d} ± 5.9	161.5^{a} ± 15.0	143.5^{b} ± 8.2	115.5^{c} ± 9.4
Unknown (MW = 210) - 2	0.0^{f} ± 0.0	$0.5^{e,f}$ ± 0.5	$3.6^{e,f}$ ± 1.3	$2.3^{e,f}$ ± 1.3	$4.4^{e,f}$ ± 0.3	11.6^{d} ± 1.8	20.9^{b} ± 1.7	27.6^{a} ± 3.7	29.5^{a} ± 1.3	5.1^{e} ± 1.2	$19.8^{b,c}$ ± 2.0	$19.6^{b,c}$ ± 1.8	$15.9^{c,d}$ ± 1.6
3-Hydroxy-5,6-dihydro-β-ionone	37.5^{f} ± 3.0	$42.9^{e,f}$ ± 1.3	$47.7^{e,f}$ ± 2.2	$51.8^{d,e}$ ± 1.3	$51.7^{d,e}$ ± 1.7	132.3^{a} ± 7.4	140.6^{a} ± 5.4	140.1^{a} ± 8.7	128.5^{a} ± 4.9	34.7^{f} ± 2.4	83.9^{b} ± 7.8	70.3^{c} ± 3.9	$64.7^{d,c}$ ± 2.0
Unknown *m/z* 119	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	5.6^{c} ± 0.7	42.7^{b} ± 2.6	58.9^{a} ± 3.9	62^{a} ± 3.7
3-Oxo-β-ionone	0.3^{d} ± 0.3	0.0^{d} ± 0.0	0.7^{d} ± 0.4	0.5^{d} ± 0.5	0.8^{d} ± 0.5	$6.9^{b,c}$ ± 0.3	11.5^{a} ± 1.1	7.6^{b} ± 1.1	5.7^{c} ± 0.3	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0
4-Hydroxy-β-ionone	7.6^{g} ± 1.2	$11.2^{e,f,g}$ ± 0.7	$15.7^{d,e}$ ± 0.6	$16.8^{c,d}$ ± 0.9	$17.4^{c,d}$ ± 0.9	24.0^{b} ± 1.9	$21.0^{b,c}$ ± 1.3	$12.9^{d,e,f}$ ± 1.4	$11.4^{e,f,g}$ ± 2.0	$14.5^{d,e,f}$ ± 1.4	58.1^{a} ± 4.1	22.4^{b} ± 1.8	$10.2^{f,g}$ ± 1.4
mixed peak includes 4-oxo-β-ionone	1.8f ± 0.3	2.8^{f} ± 0.2	2.7^{f} ± 0.2	3.0^{f} ± 0.2	2.8^{f} ± 0.1	10.2^{e} ± 0.5	50.8^{b} ± 1.1	63.9^{a} ± 5.4	61.1^{a} ± 3.5	3.5^{f} ± 0.5	17.4^{d} ± 1.8	32.7^{c} ± 3.9	35.3^{c} ± 1.2
3-Hydroxy-β-ionone	8.5^{g} ± 0.6	19.0^{e} ± 0.7	24.2^{d} ± 1.2	36.4^{c} ± 0.5	38.6^{c} ± 2.4	46.6^{b} ± 1.6	50.9^{b} ± 2.8	60.5^{a} ± 1.8	59.7^{a} ± 2.6	14.4^{f} ± 0.5	$22.4^{d,e}$ ± 1.3	$12.8^{f,g}$ ± 1.1	8.7^{g} ± 0.3
Unknown (MW = 210) - 3	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	$3.6^{d,e}$ ± 0.2	21.3^{c} ± 0.9	46.9^{a} ± 3.4	48.1^{a} ± 1.6	0.9^{e} ± 0.9	7.9^{d} ± 1.1	30.6^{b} ± 3.6	44.1^{a} ± 1.8

Table 7.5 Effect of fungal treatments on C-13 norisoprenoids in boronia marc during four day incubations. The data, which is the ratio of the peak area of diagnostic ions to the peak area of the internal standard is presented as the means and standard errors of four replicates. T- test distributions are included as superscripts.

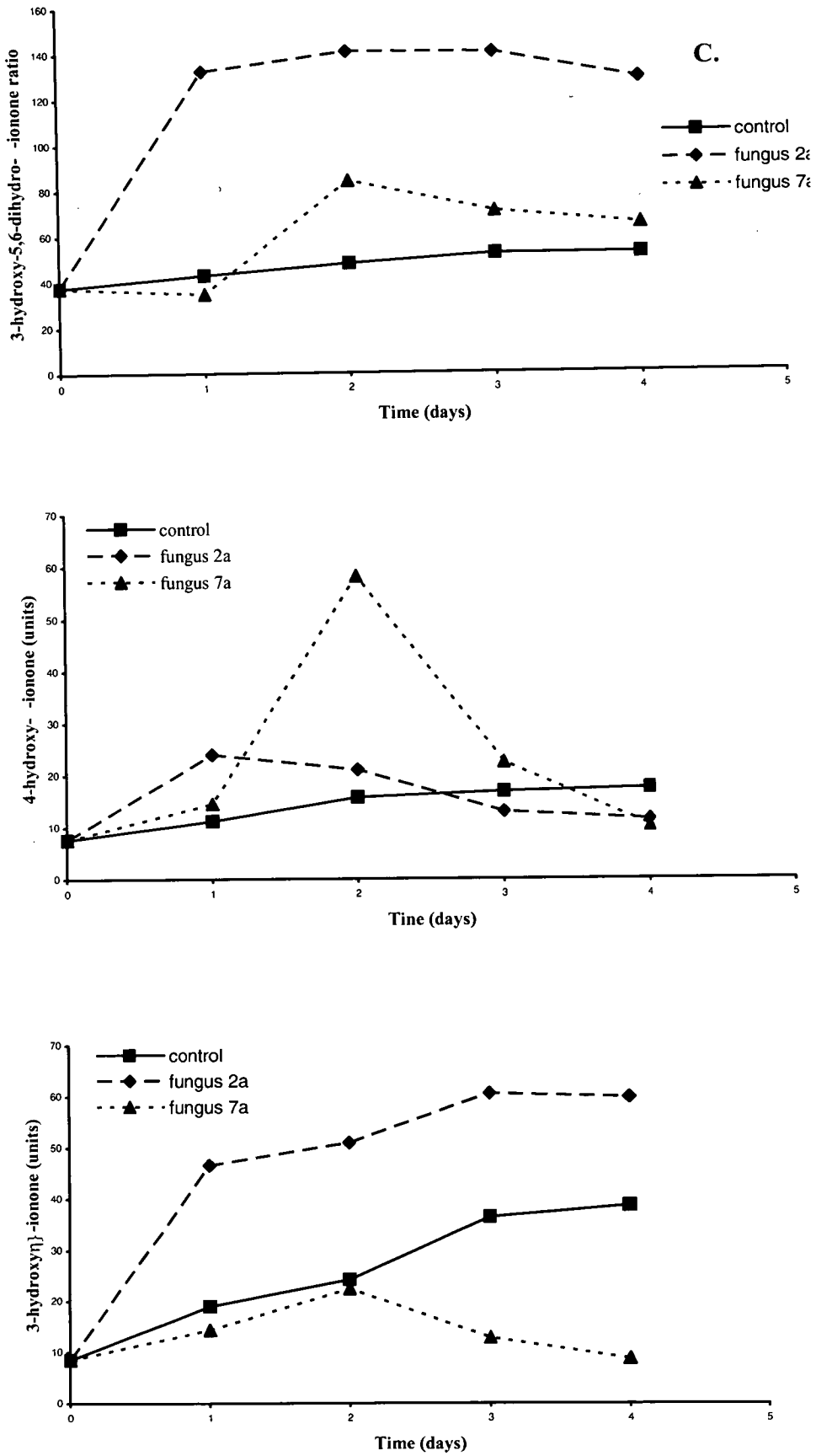


Figure 7.1 Relative changes in C-13 norisoprenoid levels during fungal incubations. **A.** 3-hydroxy-5,6-dihydro-β-ionone; **B.** 4-hydroxy-β-ionone; **C.** 3-hydroxy-β-ionone. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

It is valuable to compare the observed changes in the hydroxy C-13 norisoprenoid values to the other ionone compounds. β-Ionone and dihydroactinidiolide (not presented graphically), which is an oxidation product of β-ionone not considered to be formed biosynthetically by the plant (Weyerstahl *et al.*, 1995), did not increase due to the fungal treatments. Small but significant increases were observed in the control incubations. Treatment with fungus 2a had the highest impact on β-ionone and resulted in a decrease from the blank value of 71 to 2 units after 4 days. The reverse situation was apparent with dihydroactinidiolide where no changes were observed after treatment with fungus 2a. Treatment with fungus 7a however, resulted in a decrease in the dihydractinidiolide levels from 9 to 1 unit at 4 days. The lack of any increase in β-ionone and dihydroactinidiolide due to fungal treatments was expected as the compounds are structurally unable to form glycosides.

The results allowed some speculation as to the mechanism for the observed increases of β-ionone and the hydroxy ionones during the control incubation. The effect of heat treatment on the levels of aroma compounds in wines has been previously studied. Zocklein *et al.* (1999) found that heat treating Riesling wines at 45°C for 20 days resulted in a reduction in glycoside levels with a variable impact on the levels of volatiles. No increases in C-13 norisoprenoids that would have resulted directly from glycoside hydrolysis were noted by the authors although significant increases in vitispirane and 1,1,6-trimethyl-1,2-dihydronaphthalene were observed. A relatively newly discovered C-13 norisoprenoid, (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene was found to increase when the wine was heated to 45°C (Cox *et al.* 2005), which was suggested by the authors to be due to the presence of unknown precursor forms. In orange juice thermally induced flavour changes have been studied in relation to pasteurisation (Moshanus and Shaw, 1997). The authors observed only light changes to the levels of volatiles with some adverse olfactory changes.

The incubation temperature in this study (25°C) was lower than in the studies discussed above but this factor may still partially explain the analyte increases observed for the hydroxy ionones during the control treatment. However it does not explain the increase observed during the control incubation in relation to β-ionone as this compound cannot be structurally derived from glycosides. A possible

mechanism for the increases in control treatments may include release from cellular compartments that were previously inaccessible during petroleum ether extraction of the flowers.

Whilst there is a significant albeit small increase in dihydroactinidiolide during the control incubations it is interesting to note that the oxo analogues of the hydroxy ionones do not increase during the control treatments. **Figure 7.2** (D, E, and F) shows the relative changes that occurred to the identified oxo compounds during control and fungal treatments. The oxo compounds, which could be formed through oxidation of analogous hydroxy molecules, may not be expected to increase during control treatments if the primary route to their formation is not through release from compartments but rather through oxidation of released hydroxy ionones. Other authors have described the generation of oxidation artefacts following incubation with glycosidic enzyme preparation derived from fungal sources (Sefton and Williams, 1991). Whilst it is important not to apply the published *in vitro* observation to treatment with fungal cultures, it does suggest the need for further work.

The oxo compounds could be derived from C-40 carotenoid oxo analogues such as echinenone (Straub, 1987) or as oxidation products of the alcohol analogues. Treatment with fungus 7a gave the highest increase in 3-oxo-5,6-dihydro-β-ionone when compared to the other treatments. The relative levels of this analyte increased 27 fold from a blank of 6 to 162 units after 2 days of incubation with fungus 7a. Treatment with fungus 2a resulted in a smaller but significant increase to 45 units after 3 days. The highest increase for 3-oxo-β-ionone occurred after 2 days of treatment with fungus 2a whilst significant decreases occurred on each subsequent day. No increases were observed when the irradiated marc was treated with fungus 7a. The values for 4-oxo-β-ionone were complicated through the presence of a second analyte that had a similar mass spectrum. However when the mixed peak was considered together increases due to both fungal treatments were apparent.

The effect of fungal treatments on the relative magnitudes of the changes in 3-hydroxy-5,6-dihydro-β-ionone and 3-oxo-5,6-dihydro-β-ionone showed opposite

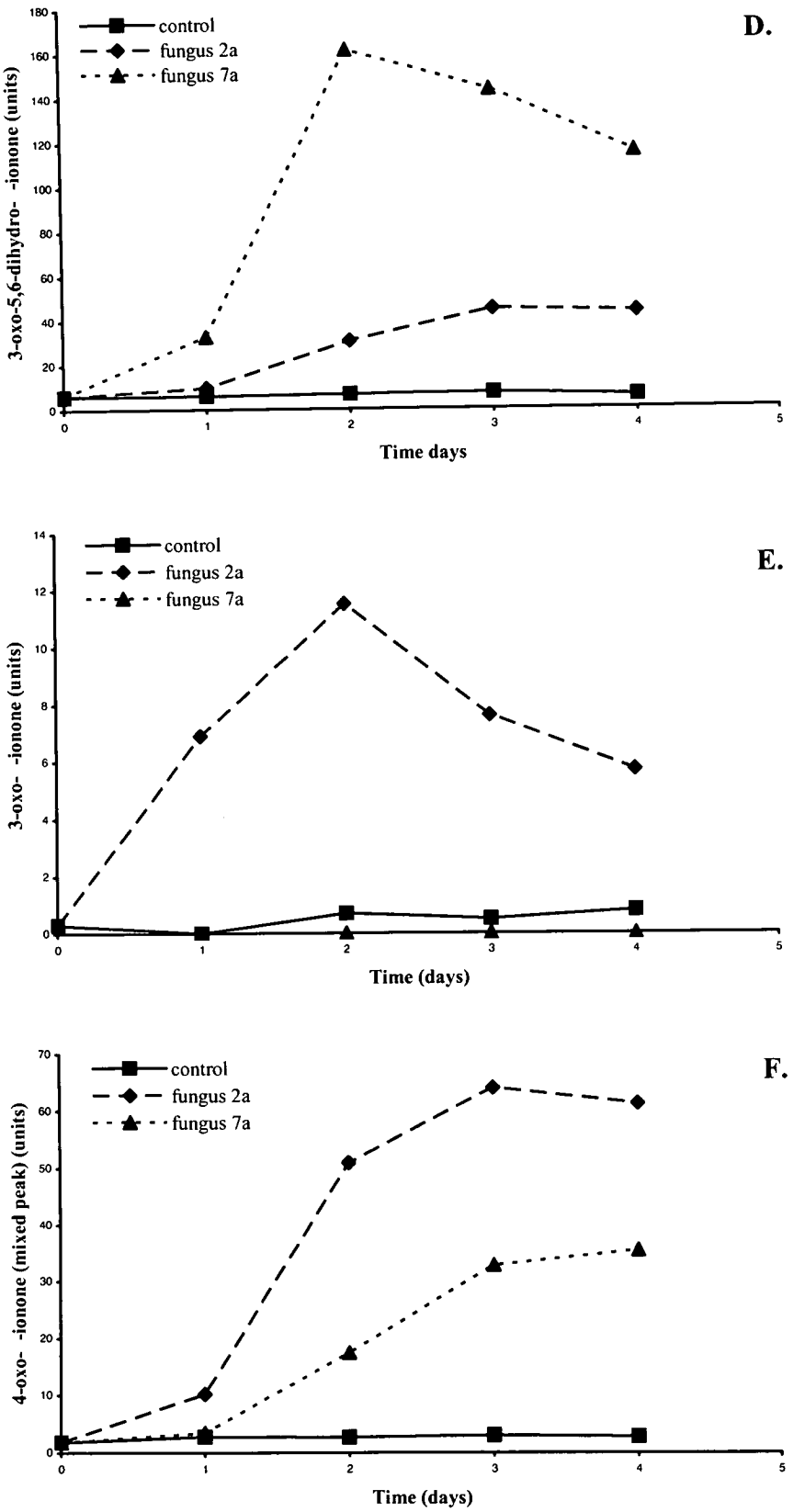


Figure 7.2 Relative changes in C-13 norisoprenoid di-ketones levels during fungal incubations. **D.** 3-oxo-5,6-dihydro-β-ionone; **E.** 3-oxo-β-ionone; **F.** 4-oxo-B-ionone (mixed peak). The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

results. A possible explanation for this is that the lower relative levels of 3-hydroxy-5,6-dihydro-β-ionone after treatment with fungus 7a (compared to high relative levels of the oxo compound) resulted from increased oxidation of this compound due to that treatment. However, the pattern was not observed when 3-hydroxy-β-ionone and 3-oxo-β-ionone were considered.

Increases in a range of unknown C-13 norisoprenoids were also observed and the changes during fungal treatment of three compounds corresponding in molecular weight to hydroxy, dihydro ionones (MW = 210) are graphically portrayed in **figure 7.3** (G, H, and I). There were no significant changes for the three unknown C-13 norisoprenoids with a molecular weight of 210 during the control treatments. However each of these compounds increased significantly during both fungal treatments. The highest increase for Unknown (MW = 210) - 1, which had a blank value of zero, occurred after three days of treatment with fungus 7a (93 units). Unknown (MW = 210) - 2 levels were higher after treatment with fungus 2a (30 units after 4 days). The third unknown (MW = 210) increased similarly from zero to 48 units after treatment with fungus 2a for 4 days, and from zero to 44 units after treatment with fungus 7a for 4 days.

The molecular weight of these compounds is consistent with them containing a hydroxyl group and therefore also consistent with their release from glycosidic conjugates. The zero blank values for these compounds and the absence of significant release during the control incubations is not inconsistent with either release from conjugates or some form of chemical / enzymatic change from a hydroxy megastigmane (MW=212). However these megastigmane ketones have not previously been reported in boronia (Weyerstahl *et al.*, 1995). Further work relating to identification of the (MW = 210) unknowns will be necessary.

Other unknown norisoprenoids were also found to increase after fungal treatments. An unknown with a molecular weight of 194, corresponding to an ionol, showed small but significant increases following fungus 7a treatment. The unknown with a characteristic *m/z* at 119, increased significantly from zero to 59 units at day 3. No increase was observed with this compound in the control or fungus 2a treatments.

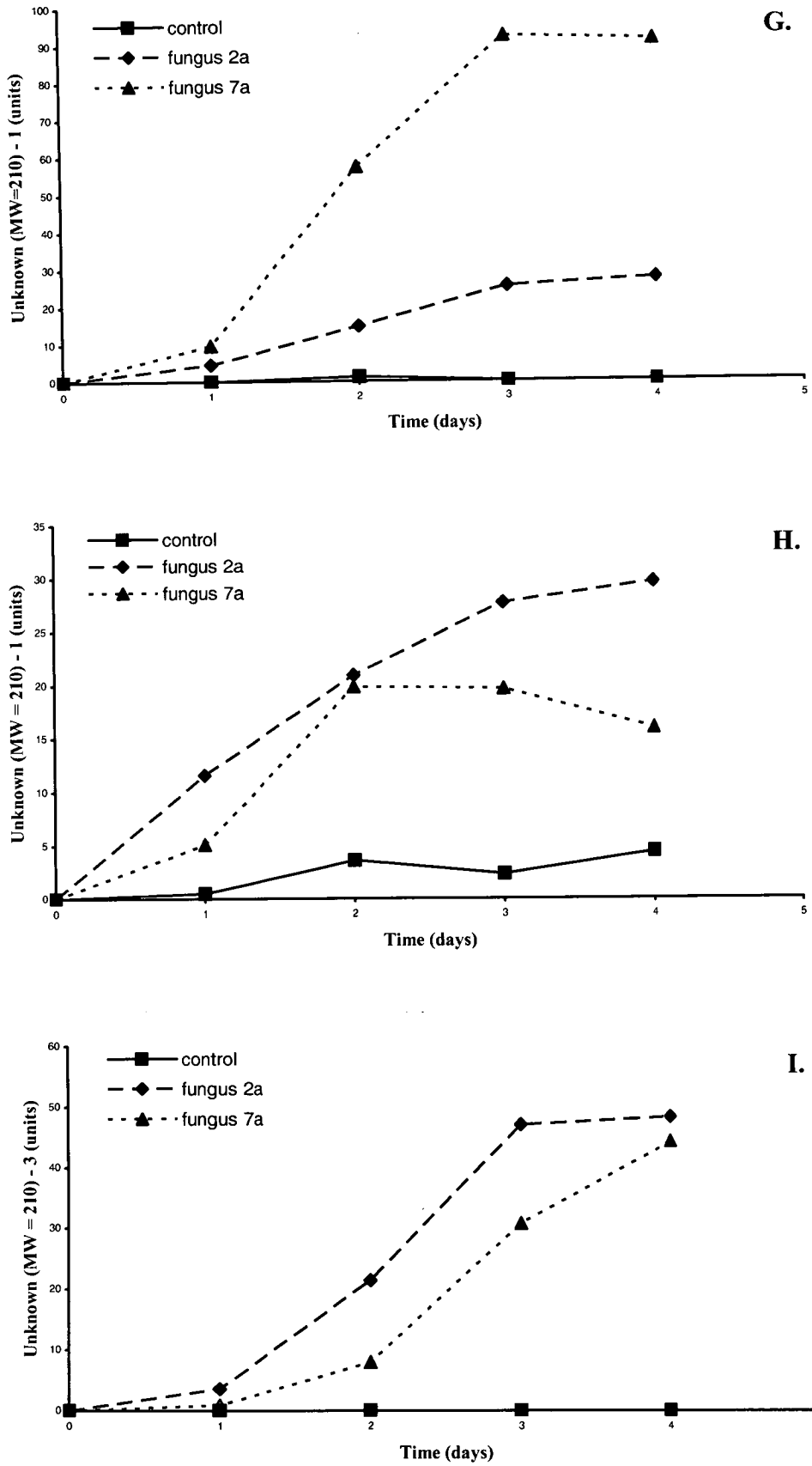


Figure 7.3 Relative changes in unknown C-13 norisoprenoid levels during fungal incubations. **G.** Unknown (MW=210) - 1; **H.** Unknown (MW=210) - 2; **I.** Unknown (MW=210) - 3. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

Monoterpenols and 8-Hydroxy Linalyl Esters

Changes in monoterpenols that occurred during fungal incubations are presented in **table 7.6** along with the t-test distribution for each compound. The highest relative increases in monoterpenols during fungal incubations were observed for 7-hydroxy hotrienol (3,7-dimethylocta-1,5-diene-3,7-diol), linalool and an unknown linalool related molecule. This data is presented graphically in **figure 7.4**. With regard to linalool there were no significant increases during the control incubations. However both fungal treatments showed significant increases in linalool after two days with levels increasing from 0.3 to 15 units for fungus 2a and to 29 units for fungus 7a which represented a 100 fold increase. In addition, for fungus 7a significant decreases in linalool after the day 2 peak to 19 units at day 3 and 12 units at day 4 were noted. No statistically significant decreases were observed during treatment with fungus 2a in that time period.

As the stability of aroma and flavour volatiles following release from the conjugated form is critical with regard to selecting the length of incubation and linalool is a floral volatile likely to have a favourable impact on the boronia extract, it is worth discussing the changes observed with linalool. Possible reasons for the observed treatment specific decrease in linalool after 2 days may be (1) instability in the conditions formed during incubation or (2) metabolism by the fungi during the incubation process. In stability studies on monoterpenes it was demonstrated that linalool was more susceptible to oxidative processes than β-pinene, citral, limonene and β-myrcene (Bertolini *et al.*, 2001).

Bazemore *et al.* (2003) referred to linalool as a reactive molecule. Stability studies with regard to pH by other authors (Baxter *et al.*, 1978; as cited by Bazemore *et al.*, 2003) indicated treatment with citric acid at 24°C for 20 days showed a 56% reduction in linalool with matching increases in α-terpineol and 3,7-dimethylocta-1-ene-3,7-diol. Whilst no measurements of pH changes were taken during this experiment it should be noted that fungal treatments were conducted over a 4 day period (1/5 the time of the quoted experiment). Temperature alone is unlikely to be the reason for the observed decrease in linalool during incubation with fungus7a as no significant decrease in linalool was observed during incubation with fungus 2a. It is possible that the different fungal isolates are demonstrating a different potential for

	Blank	Control				Fungus 2a				Fungus 7a			
Time (days)	0	1	2	3	4	1	2	3	4	1	2	3	4
Compound													
Monoterpenols													
Linalool	0.3^{f} ± 0.3	0.0^{f} ± 0.00	1.1^{f} ± 0.6	1.6^{f} ± 0.6	1.0^{f} ± 0.6	$8.0^{d,e}$ ± 1.4	$15.0^{b,c}$ ± 1.3	$9.8^{c,d,e}$ ± 2.4	$10.4^{c,d}$ ± 1.4	$4.2^{e,f}$ ± 1.2	29.2^{a} ± 5.2	18.9^{b} ± 1.9	$11.7^{c,d}$ ± 2.2
Pinocarveol *	$3.9^{c,d}$ ± 0.5	$3.7^{c,d}$ ± 0.4	3.1^{d} ± 0.5	5.3^{c} ± 0.7	3.1^{d} ± 0.3	8.5^{b} ± 0.7	10.9^{a} ± 0.3	$9.2^{a,b}$ ± 1.5	$9.7^{a,b}$ ± 1.2	$3.7^{c,d}$ ± 0.8	$4.8^{c,d}$ ± 0.4	$4.1^{c,d}$ ± 0.3	5.8^{c} ± 0.6
Verbenol *	$2.3^{b,c}$ ± 0.5	$1.6^{c,d}$ ± 0.5	0.6^{d} ± 0.4	$1.7^{c,d}$ ± 0.6	$1.5^{c,d}$ ± 0.5	$2.4^{b,c}$ ± 0.3	$3.6^{a,b}$ ± 0.2	4.1^{a} ± 0.5	4.5^{a} ± 0.5	$1.8^{c,d}$ ± 0.4	$3.4^{a,b}$ ± 0.7	$3.5^{a,b}$ ± 0.4	4.3^{a} ± 0.4
Borneol *	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	3.2^{c} ± 0.1	9.9^{b} ± 0.5	12.9^{a} ± 1.3	13.7^{a} ± 0.8	0.0^{e} ± 0.0	1.7^{d} ± 0.4	$2.1^{c,d}$ ± 0.3	3.3^{c} ± 0.4
7-Hydroxy-hotrienol	10.7^{f} ± 1.2	$16.3^{e,f}$ ± 0.8	19.7^{e} ± 0.4	21.5^{e} ± 1.0	19.5^{e} ± 0.9	32.0^{d} ± 2.1	60.1^{c} ± 1.9	65.2^{c} ± 4.0	$66.6^{b,c}$ ± 2.3	$17.2^{e,f}$ ± 2.2	$74.0^{a,b}$ ± 3.8	81.2^{a} ± 5.2	79.6^{a} ± 4.7
Myrtenol *	4.0^{d} ± 1.0	4.1^{d} ± 0.8	$3.3^{d,e}$ ± 0.6	$4.8^{c,d}$ ± 0.6	$3.4^{d,e}$ ± 0.4	$6.8^{a,b,c}$ ± 0.3	$7.7^{a,b}$ ± 0.3	9.1^{a} ± 1.3	9.2^{a} ± 0.6	$5.5^{b,c,d}$ ± 1.3	$1.3^{e,f}$ ± 0.1	0.22^{f}	0.0^{f} ± 0.0
unknown linalool related	3.4^{d} ± 0.1	4.2^{d} ± 0.2	4.9^{d} ± 0.4	6.3^{d} ± 0.3	5.6^{d} ± 0.1	75.2^{c} ± 3.3	127.4^{a} ± 5.6	$117.5^{a,b}$ ± 19.2	94.4^{c} ± 11.1	74.4^{c} ± 8.6	127.6^{a} ± 6.4	$95.4^{b,c}$ ± 6.9	$95.0^{b,c}$ ± 4.6
8-hydroxy-linalool	$34.5^{f,g,h}$ ± 6.2	$58.2^{b,c,d}$ ± 3.3	$68.3^{a,b}$ ± 2.6	78.3^{a} ± 3.2	$67.3^{a,b,c}$ ± 4.7	$46.8^{d,e,f}$ ± 5.4	$52.5^{c,d,e}$ ± 3.8	$30.3^{g,h}$ ± 7.4	$21.8^{h,i}$ ± 5.9	$38.8^{e,f,g}$ ± 7.4	$31.6^{f,g,h}$ ± 8.0	13.6^{i} ± 4.7	7.3^{i} ± 2.7
8-Hydroxy linalyl esters													
8-Hydroxy linalyl octanoate	$60.9^{a,b}$ ± 12.3	$58.4^{a,b}$ ± 2.7	65.9^{a} ± 4.8	61.5^{a} ± 3.3	49.3^{b} ± 2.5	21.0^{c} ± 3.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	26.5^{c} ± 5.1	1.4^{d} ± 0.9	0.0^{d} ± 0.0	0.0^{d} ± 0.0
8-Hydroxy linalyl nonanoate	36.0^{b} ± 9.5	37.9^{b} ± 3.5	49.1^{a} ± 5.4	$44.8^{a,b}$ ± 2.0	39.9^{b} ± 2.6	19.6^{c} ± 3.4	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	14.4^{c} ± 1.1	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0d ± 0.0
8-Hydroxy linalyl decanoate	$61.0^{c,d}$ ± 18.4	$70.3^{b,c}$ ± 3.7	95.2^{a} ± 7.2	91.9^{a} ± 6.2	$86.1^{a,b}$ ± 5.5	43.4^{d} ± 5.0	3.8^{e} ± 0.5	0.0^{e} ± 0.0	0.0^{e} ± 0.0	13.9^{e} ± 3.1	1.1^{e} ± 1.1	0.0^{e} ± 0.0	0.0^{e} ± 0.0
8-Hydroxy linalyl undecanoate	5.9^{b} ± 2.5	8.0^{b} ± 0.3	11.0^{a} ± 0.8	11.6^{a} ± 0.6	10.8^{a} ± 1.0	6.4^{b} ± 0.8	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.9^{c} ± 0.6	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.1^{c} ± 0.1

Table 7.6 Effect of fungal treatments on monoterpenols and linalyl esters in boronia marc over four days of incubation. The data, which is the ratio of the peak area of diagnostic ions to the peak area of the internal standard is presented as the means and standard errors of four replicates. T- test distributions are included as superscripts. * Compounds not previously identified in boronia (Weyerstahl *et al.*, 1995).

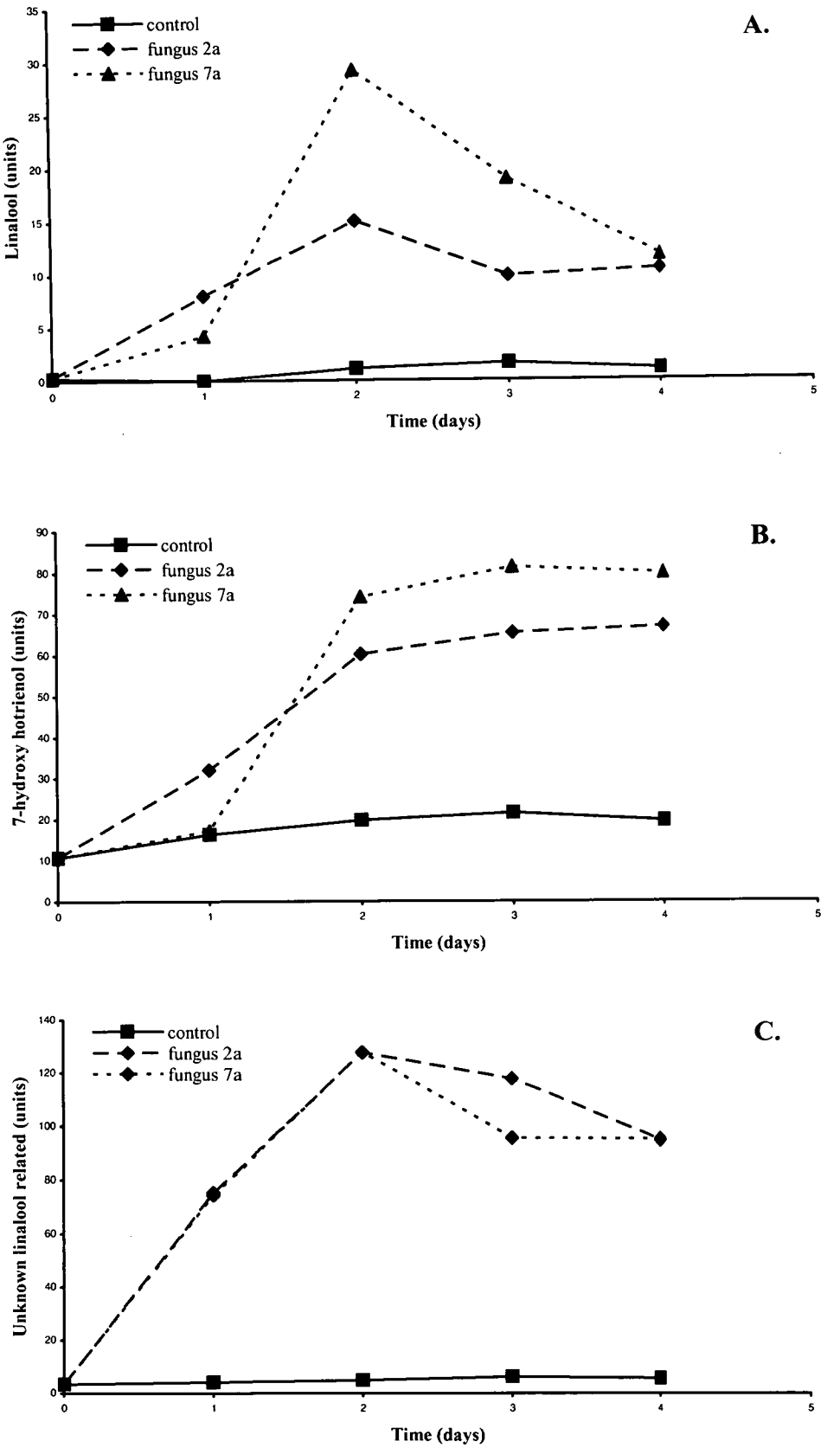


Figure 7.4 Relative changes in monoterpenol levels during fungal incubations. **A.** linalool; **B.** 7-hydroxy hotrienol; **C.** Unknown linalool related. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

metabolism of released volatiles. Similar arguments as discussed for linalool may be applicable to changes in other monoterpenols during incubation treatments.

Large increases were also observed between 0 and 2 days for both the unknown linalool related compound and 7-hydroxy hotrienol through treatment with both fungal isolates. The level of the unknown linalool related compound showed a 37 fold increase from a blank result of 3 to 127 units for fungus 2a and 128 units for fungus 7a after 2 days of treatment. Similarly to the observation with linalool during treatment with fungus 7a, levels of the unknown compound reduced significantly after day 2 during both fungal treatments. There were no significant increases for this compound during the control incubation. The monoterpenediol, 7-hydroxy hotrienol was observed to increase from a blank level of 11 to 60 units after 2 days of treatment with fungus 2a and to 74 units after two days of incubation with fungus 7a. Longer incubation periods with the fungal isolates did not result in any further significant changes to the level of 7-hydroxy hotrienol. Additionally, 7-hydroxy hotrienol was observed to increase in the control incubation from 11 to 20 units after two days at 25°C.

Changes in the levels of four bicyclic monoterpenols (pinocarveol, verbenol, borneol and myrtenol), not reported as present in boronia by Weyerstahl *et al.* (1995), were also observed. Borneol was not present in the blank or in control treatments but did increase significantly during both fungal treatments rising to 14 units at day four of treatment with fungus 2a. The possible role of fungal metabolism, other than glycosidase action, in the biosynthesis of these compounds was discussed in the previous section.

The 8 hydroxy linalyl esters, the data for which is presented **in table 7.6**, were metabolised rapidly by both fungal treatments. All four esters were reduced to zero at day 3 with only very small amounts remaining in some cases at day 2. However significant increases in the nonanoate, decanoate and undecanoate esters were observed in the control incubations after two days at 25°C. It might have been expected that metabolism of these compounds would release 8-hydroxy linalool. However the changes with regard to 8-hydroxy linalool indicated a more complex pattern. Whilst 8-hydroxy linalool increased during the control incubation from the

blank level of 35 to a highest level of 78 units at 3 days, no significant increases were observed during the fungal treatments. Furthermore this compound decreased during the incubation period to 22 units at day 4 for fungus 2a and to 7 units at day 4 for fungus 7a. These observations with regard to 8-hydroxy linalool indicated that this compound is either not being generated from the linalyl esters or that any generation from hydrolysis of the esters is being offset by fungal metabolism of the compound. It is also possible that the unknown linalool related compound may have been generated from the linalyl esters.

Cinnamates

Similarly to the 8-hydroxy linalool esters, the cinnamate molecules which are found in boronia have a monoterpene moiety as part of the molecule. They were earlier determined through GC-olfactometry as possible contributors to the aroma profile of boronia. Hence they were examined with regard to the impact of the fungal treatments and the data showing the results of the statistical analysis is presented in **table 7.7**.

The methyl cinnamates discussed here are bound to either geranyloxy or 5-hydroxy geranyloxy moieties. The geranyloxy cinnamates showed similar patterns with regard to the experimental treatments and the data for methyl (Z)-4-(geranyloxy)cinnamate is presented in **figure 7.5 A**. Both compounds showed small but significant increases during the control incubations but appeared to remain unchanged following treatment with fungus 2a. However, the absence of any increase comparable to the control treatment may indicate that the fungal treatment resulted in some metabolism of these compounds. Treatment with fungus 7a however resulted in significant disappearance of both geranyloxy cinnamates (**see figure 7.5 B and C**). The (5-hydroxy)geranyloxy cinnamates did not change significantly during the control treatment. However both fungal treatments resulted in large decreases in these compounds over the four-day period of the incubation with similar patterns of disappearance apparent.

		Control				Fungus 2a				Fungus 7a			
Time (days)	**0**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Compounds													
Cucurbates													
Methyl diepicucurbate	1.8^{g} ± 0.6	$3.3^{f,g}$ ± 0.3	$6.6^{e,f}$ ± 0.8	$7.3^{d,e}$ ± 0.5	$8.6^{d,e}$ ± 0.7	13.2^{c} ± 1.7	$7.1^{d,e}$ ± 0.4	$6.6^{e,f}$ ± 1.4	$6.5^{e,f}$ ± 1.1	$10.7^{c,d}$ ± 2.0	28.5^{a} ± 2.0	$26.6^{a,b}$ ± 1.8	23.3^{b} ± 1.1
Methyl cucurbate	$0.8^{g,h}$ ± 0.3	0.0^{h} ± 0.0	$1.3^{f,g,h}$ ± 0.8	$2.2^{e,f,g}$ ± 0.8	$2.8^{d,e,f}$ ± 0.5	5.4^{c} ± 0.7	$4.2^{d,c}$ ± 0.3	$3.2^{d,e}$ ± 0.4	$3.2^{d,e}$ ± 0.4	$3.9^{c,d,e}$ ± 0.8	10.5^{a} ± 0.7	8.1^{b} ± 0.9	7.8^{b} ± 0.6
Methyl epicucurbate	2.3^{e} ± 0.3	$3.9^{d,e}$ ± 0.2	$5.3^{c,d,e}$ ± 0.8	$5.2^{c,d,e}$ ± 0.3	$5.5^{b,c,d}$ ± 0.5	8.5^{b} ± 0.7	$7.8^{b,c}$ ± 0.7	$5.6^{b,c,d}$ ± 0.9	$5.2^{c,d,e}$ ± 0.8	8.5^{b} ± 2.0	19.6^{a} ± 2.1	19.5^{a} ± 1.6	16.8a ± 1.0
Tiglamides													
N-[2-(4-prenyloxyphenyl)]ethyl tiglamide	826.1^{a} ± 145.5	$628.4^{b,c}$ ± 18.5	827.8^{a} ± 91.7	$704^{a,b,c}$ ± 17.1	$642.8^{b,c}$ ± 32.5	$590.7^{b,c}$ ± 40.8	237.9^{d} ± 41.9	$107.9^{d,e}$ ± 13.7	58.3^{e} ± 16.5	$725.7^{a,b}$ ± 43.2	545.8^{c} ± 43.6	$671^{a,b,c}$ ± 59.4	$644.1^{b,c}$ ±51.6
Unknown (*m/z* 120, 204)	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	26.5^{c} ± 2.0	51.8^{b} ± 3.7	59.8^{a} ± 6.2	48.6^{b} ± 3.1	0.0^{d} ± 0.0	0.0^{d} ± 0.0	$0.6.0^{d}$ ± 0.6	1.0^{d} ± 0.6
Unknown (*m/z* 120, 99, 218))	$17.5^{a,b}$ ± 9.1	$27.7^{a,b}$ ± 2.3	37.2^{a} ± 2.8	$33.3^{a,b}$ ± 1.6	$17.5^{a,b}$ ± 6.4	$17.5^{a,b}$ ± 7.7	$17.6^{a,b}$ ± 7.1	$20.2^{a,b}$ ± 10.0	15.8^{b} ± 6.8	$18.4^{a,b}$ ± 8.8	$18.6^{a,b}$ ± 8.8	$17.7^{a,b}$ ± 7.5	16.1^{b} ± 7.1
Cinnamates													
Methyl (Z)-4-(geranyloxy)cinnamate	$62.2^{b,c,d}$ ± 19.5	$76.9^{b,c}$ ± 4.3	150.1^{a} ± 14.0	160.9^{a} ± 7.0	145.8^{a} ± 13.1	92.1^{b} ± 19.5	89.2^{b} ± 8.0	$84.4^{b,c}$ ± 11.3	$74.8^{b,c}$ ± 7.5	$71.5^{b,c}$ ± 11.5	$55.5^{c,d}$ ± 8.9	$38.0^{d,e}$ ± 2.1	22.2^{e} ± 2.3
Methyl (E)-4-(geranyloxy)cinnamate	$75.2^{b,c}$ ± 23.0	$73.6^{b,c}$ ± 3.6	122.4^{a} ± 10.8	127.2^{a} ± 4.8	118.9^{a} ± 11.6	$99.2^{a,b}$ ± 19.0	$99.1^{a,b}$ ± 14.4	$67.1^{b,c}$ ± 8.9	$73.5^{b,c}$ ± 10.9	65.1^{c} ± 7.8	$46.2^{c,d}$ ± 5.1	27.7^{d} ± 1.1	19.2^{d} ± 1.6
Methyl (Z)-4-(5-hydroxy-geranyloxy)cinnamate	$297.1^{a,b}$ ± 55.2	$280^{a,b,c}$ ± 6.7	322.2^{a} ± 22.0	$291^{a,b,c}$ ± 9.7	$254^{b,c,d}$ ± 15.0	223.5^{d} ± 22.7	138.4^{e} ± 12.2	$80.2^{f,g}$ ± 8.7	56.7^{g} ± 9.1	$236^{c,d}$ ± 7.4	$125.5^{e,f}$ ± 3.2	$74.7^{f,g}$ ± 6.6	36.1^{g} ± 5.1
Methyl (E)-4-(5-hydroxy-geranyloxy)cinnamate	$521^{a,b}$ ± 96.4	$423^{b,c,d}$ ± 18.3	543.5^{a} ± 47.0	$475^{a,b,c}$ ± 6.5	$421.6^{c,d}$ ± 21.4	$399.9^{c,d}$ ± 43.0	201.3^{e} ± 23.1	$88.7^{f,g}$ ± 12.1	$61.1^{f,g}$ ± 11.8	370.9^{d} ± 13.6	$134.8^{e,f}$ ± 8.9	$50.7^{f,g}$ ± 4.2	28.0^{g} ± 3.7

Table 7.7 Effect of fungal treatments on cucurbates, tiglamides and cinnamates in boronia marc over four days of incubation. The data, which is the ratio of the peak area of diagnostic ions to the peak area of the internal standard is presented as the means and standard errors of four replicates. T- test distributions are included as superscripts.

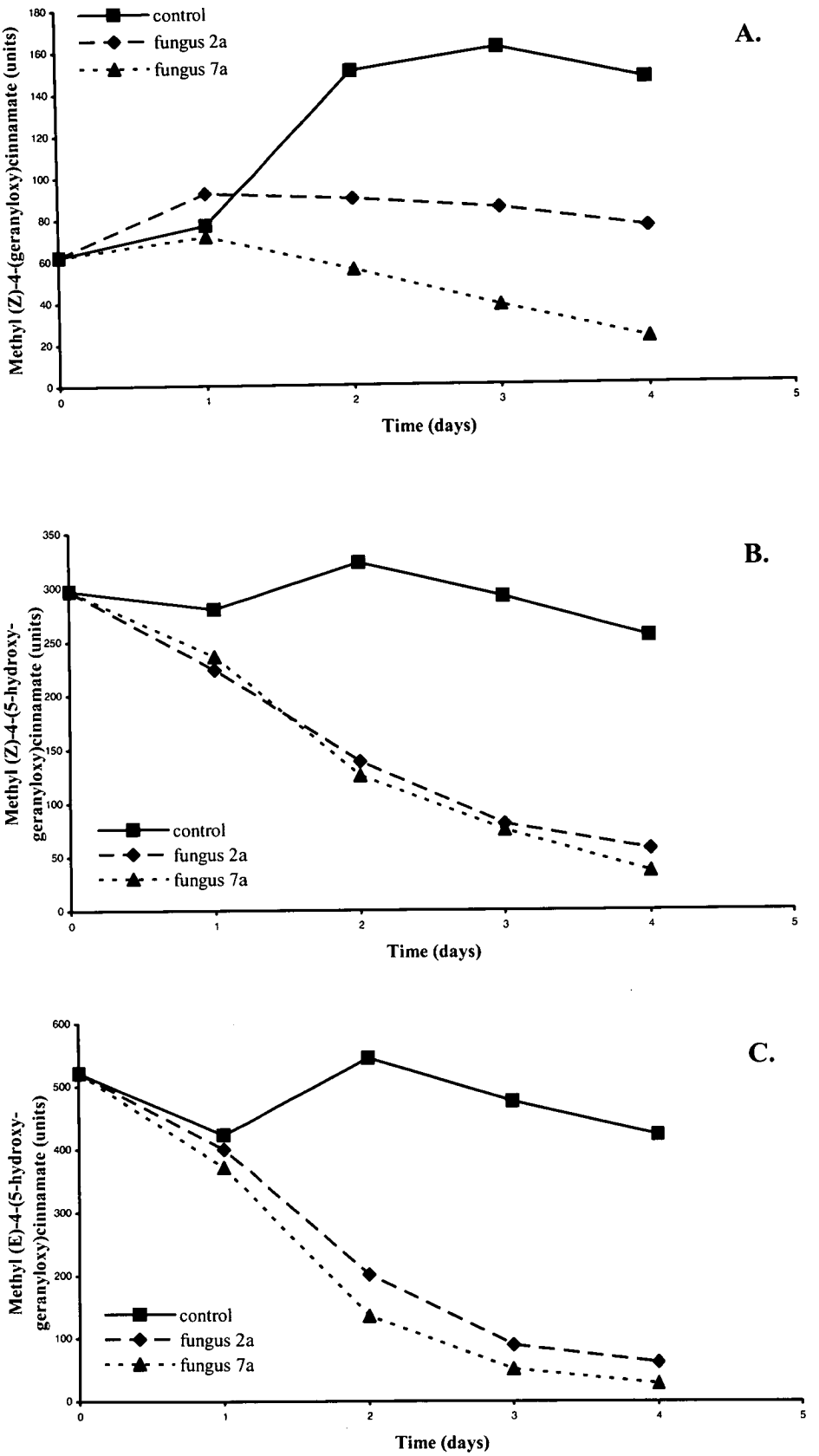


Figure 7.5 Relative changes in the levels of cinnamate compounds during fungal incubations. **A.** Methyl (Z)-4-(geranyloxy)cinnamate; **B.** Methyl (Z)-4-(5-hydroxy-geranyloxy)cinnamate; **C.** Methyl (E)-4-(5-hydroxygeranyloxy)cinnamate. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

Cinnamate esters have previously been noted as making a significant contribution to the aroma profile of products derived from plants. In Muscat wine (Etievant *et al.*, 1983), ethyl cinnamate was rated very highly in its contribution to wine aroma. Although the aroma threshold was an order of magnitude lower than β-ionone, the study additionally determined that this compound had a low aroma threshold in comparison to most other compounds present in the wine. Descriptions of the aroma of ethyl cinnamate vary from flowery (Culleré *et al.*, 2003) to strawberry cream (López *et al.*, 1999), strawberry/sweet (Lermusieau *et al.*, 2001) and fruity, banana passa-like (Miranda *et al.*, 2001) thus implying a range of interpretations. Aroma descriptions of other cinnamate esters include flowery for ethyl dihydrocinnamate (Culleré *et al.*, 2003) and sweet for methyl cinnamate (Buettner and Welle, 2004).

Cucurbates

The results for the effect of the control and fungal treatments on methyl cucurbates are presented in **table 7.7**. All three cucurbate compounds detected in boronia marc were similarly impacted by the treatments as is apparent from the graphical presentation in **figure 7.6**. The control treatments for each cucurbate resulted in significant increases over the 4 day incubation period when compared to the blank. Methyl diepicucurbate increased from a blank of 2 to 9 units at 4 days representing a 5 fold increase. Treatment with fungus 7a resulted in the three cucurbate levels reaching a maximum level after 2 days. Methyl diepicucurbate had the largest increase from 2 to 29 units after 2 days. The largest increases during treatment with fungus 2a were after 1 day with methyl diepicucurbate rising from a blank of 2 to 13 units after incubation for 1 day. Decreases in the cucurbate levels were observed on subsequent days.

The methyl cucurbates are the alcohol analogues of the methyl jasmonates. However, unlike the jasmonates, of which the (3R,7S)-methyl jasmonate isomer is reported to have a strong lemon-like odour (Gansser *et al.*, 1997), no details with regard to the olfactory properties of these compounds were available in the literature. There is however increasing interest in the possible analogous role that methyl jasmonates and methyl cucurbates have with regard to action as plant hormones (Seto *et al.*, 1999).

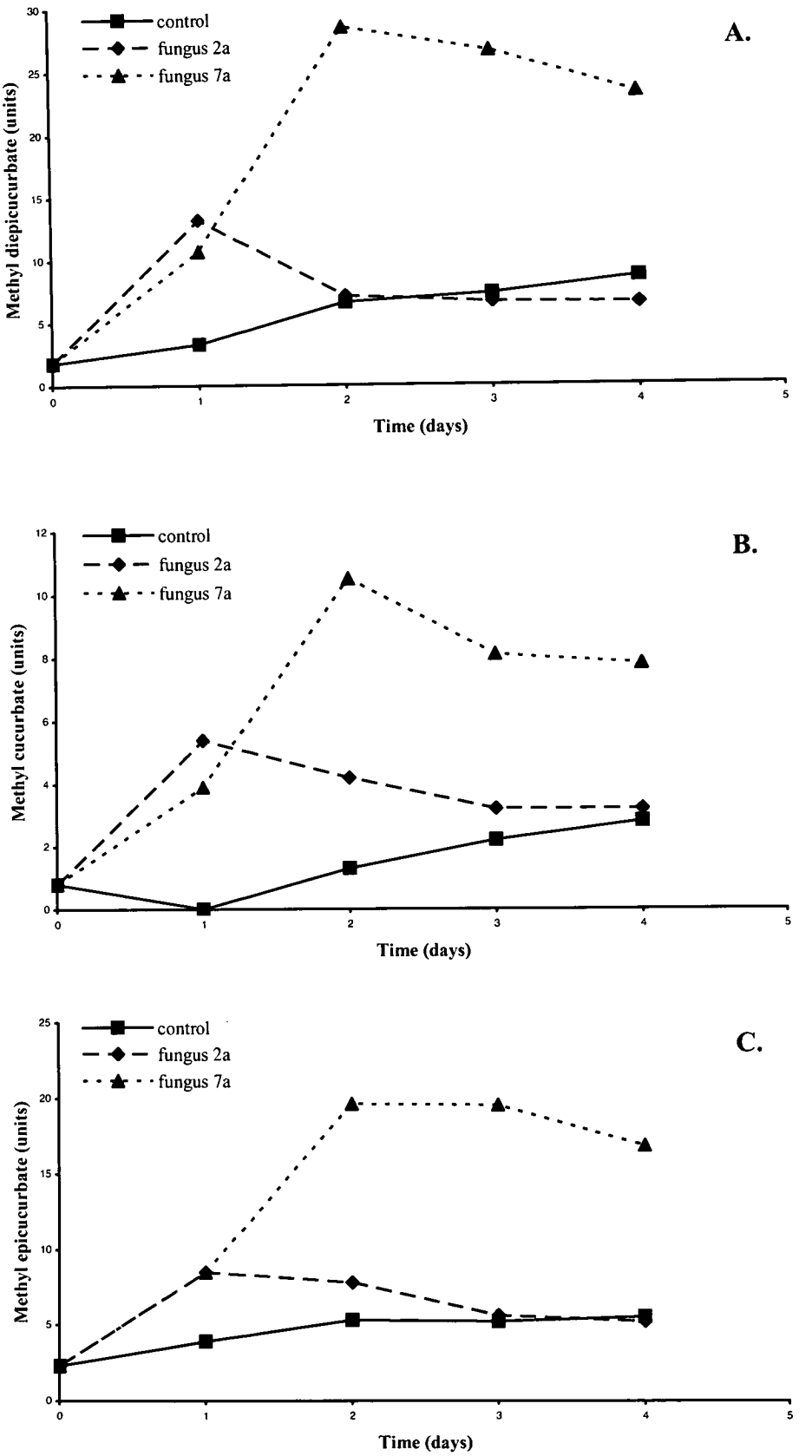


Figure 7.6 Relative changes in the levels of cucurbates during fungal incubations. **A.** Methyl diepicucurbate; **B.** Methyl cucurbate; **C.** Methyl epicucurbate. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

Tiglamides

Two tiglamides, N-[2-(4-hydroxyphenyl)ethyl] tiglamide and N-[2-(4-prenyloxyphenyl)ethyl] tiglamide have been identified in boronia (Weyerstahl *et al.*, 1995). In this study changes in the levels of N-[2-(4-prenyloxyphenyl)ethyl] tiglamide and two unknown compounds with an mass ion peak of *m/z* = 120, which is characteristic of the nitrogen containing tiglamides, were determined and the results are presented in **table 7.7** and **figure 7.7 A**. Treatment with fungus 2a resulted in a decrease in the levels of N-[2-(4-prenyloxyphenyl)ethyl] tiglamide from a blank value of 826 to 58 units after 4 days of incubation at 25°C. This decrease is matched by the pattern of appearance of the proposed unknown tiglamide, with mass spectral peaks at *m/z* 120 and 204, following treatment with fungus 2a. Treatment with fungus 7a resulted in a small significant decrease in N-[2-(4-prenyloxyphenyl)ethyl] tiglamide at 4 days compared to the blank but that was not significantly different to the control at 4 days. No significant changes were observed for the proposed unknown tiglamides following control and fungus 7a treatments.

Tiglamides were not found to be mentioned in the literature with regard to flavour and aroma. However tiglates, which are the ester equivalents of the tiglamides, have been previously identified in boronia (Weyerstahl *et al.*, 1995) and guava fruit from the Reunion Islands (Vernin *et al.*, 1998). These compounds were described by the later authors as contributing to the fruity notes in guava aroma.

Other Compounds

Table 7.8 details data on a range of further compounds measured during this time trial experiment. Changes to the levels of two shikimate compounds not previously identified in the boronia extract were or particular interest. The floral shikimate volatile, 2-phenylethanol (see **figure 7.8 A**), increased by 27 times after 1 day of treatment with fungus 7a. This compound increased 22 fold after two days of treatment with fungus 2a. Both treatments resulted in significant reductions in 2-phenylethanol levels following peak concentrations with levels falling to 0.6 after 4 days of treatment with fungus 7a. The other shikimate derived volatile, 4-vinylphenol, which has been described as having an almond shell aroma (Culleré *et*

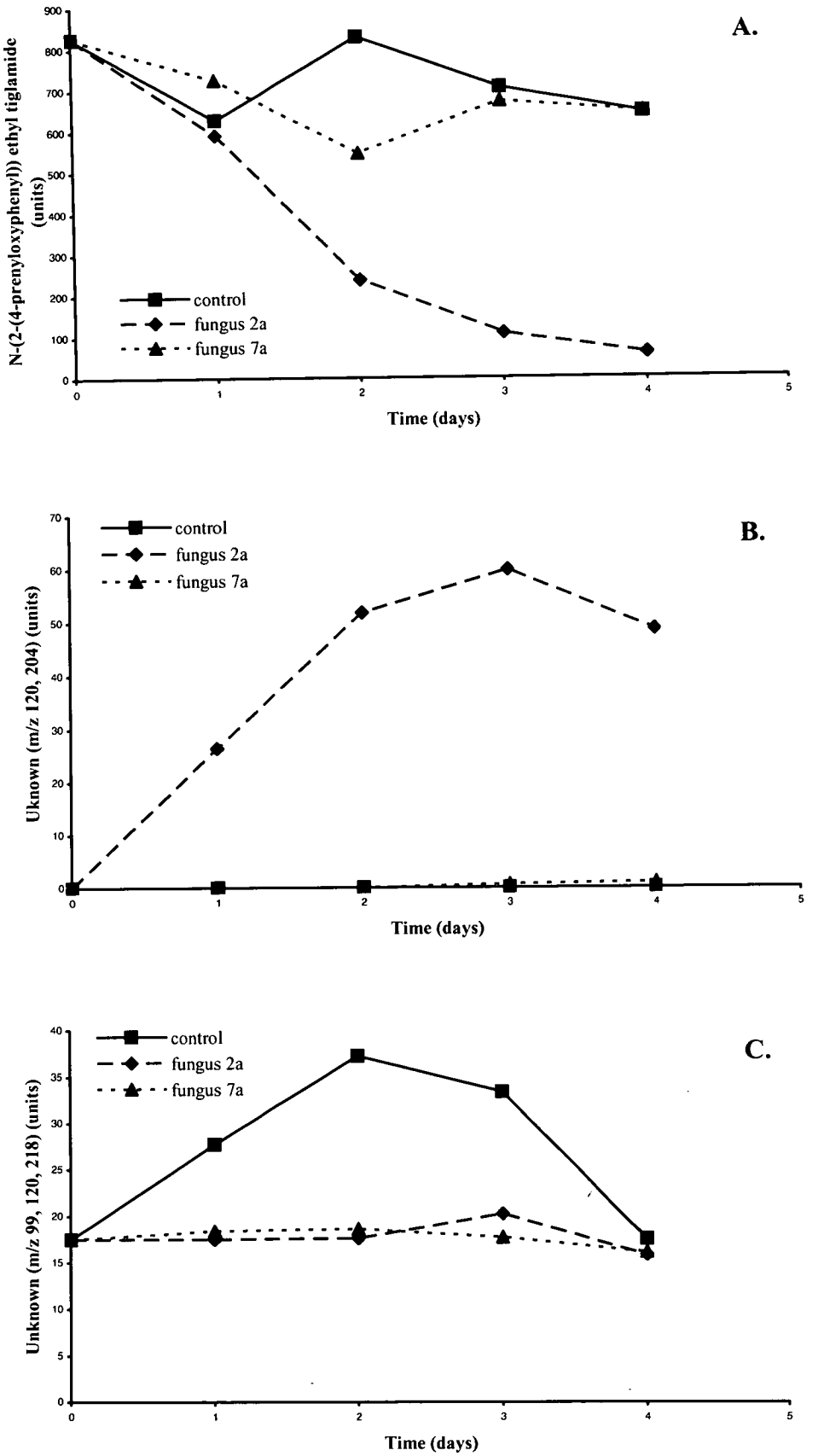


Figure 7.7 Relative changes in the levels of tiglamides during fungal incubations. **A.** N-(2-(4-prenyloxyphenyl)) ethyl tiglamide; B. Unknown (m/z 120, 204) ; **C.** Unknown (m/z 99, 120, 218). The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

		Control				Fungus 2a				Fungus 7a			
Time (days)	0	1	2	3	4	1	2	3	4	1	2	3	4
Compound													
Shikimates													
2-Phenylethanol	1.1^{e} ± 0.2	1.6^{e} ± 0.2	2.5^{e} ± 0.2	3.4^{e} ± 0.3	3.6^{e} ± 0.2	$20.6^{b,c}$ ± 1.3	$24.1^{a,b}$ ± 1.6	$16.1^{c,d}$ ± 1.8	$16.3^{c,d}$ ± 1.1	30.1^{a} ± 7.6	13.3^{d} ± 3.4	4.1^{e} ± 0.5	0.6^{e} ± 0.4
4-Vinylphenol	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	5.2^{a} ± 2.0	$3.4^{a,b}$ ± 1.5	2.4^{b} ± 0.8	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0
Other Compounds													
β-Caryophyllene	0.6^{g} ± 0.6	$2.4^{e,f,g}$ ± 0.2	$4.4^{b,c,d}$ ± 0.5	$5.5^{b,c}$ ± 0.3	$4.1^{c,d,e}$ ± 0.5	$2.1^{f,g}$ ± 0.8	$2.5^{e,f}$ ± 0.3	$2.6^{d,e,f}$ ± 0.2	$2.5^{e,f}$ ± 0.2	$1.8^{f,g}$ ± 0.8	$3.4^{d,e,f}$ ± 1.2	6.2^{b} ± 0.6	10.0^{a} ± 0.3
Dodecanol	7.7^{c} ± 1.3	14.4^{a} ± 1.2	14.6^{a} ± 0.4	15.5^{a} ± 0.5	10.9^{b} ± 1.5	3.1^{d} ± 1.5	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0	6.4^{c} ± 0.7	0.0^{e} ± 0.0	0.0^{e} ± 0.0	0.0^{e} ± 0.0
Spathulenol	$3.2^{d,e,f}$ ± 0.3	$4.1^{b,c,d}$ ± 0.3	$4.2^{a,b,c}$ ± 0.4	5.2^{a} ± 0.4	$4.2^{a,b,c}$ ± 0.2	$4.4^{a,b}$ ± 0.2	$3.5^{b,c,d,e}$ ± 0.4	$3.3^{d,e,f}$ ± 0.2	$2.7^{e,f}$ ± 0.4	$3.5^{b,c,d,e}$ ± 0.2	2.5^{f} ± 0.5	$2.9^{e,f}$ ± 0.1	$3.4^{c,d,e,f}$ ± 0.5
Dodecyl acetate	$2.9^{b,c}$ ± 0.6	$3.4^{a,b,c}$ ± 0.3	$3.8^{a,b}$ ± 0.5	3.8^{a} ± 0.4	$2.7^{c,d}$ ± 0.3	1.9^{d} ± 0.3	0.3^{e} ± 0.1	0.0^{e} ± 0.0	0.0^{e} ± 0.0	1.8^{d} ± 0.5	0.4^{e} ± 0.1	0.0^{e} ± 0.0	0.0^{e} ± 0.0
Heptadec-8-ene	$14.9^{b,c}$ ± 1.0	$18.0^{a,b}$ ± 1.1	20.5^{a} ± 0.7	20.3^{a} ± 1.1	$18.4^{a,b}$ ± 0.7	$18.7^{a,b}$ ± 1.3	$11.1^{c,d}$ ± 1.8	$10.0^{d,e}$ ± 1.9	$7.5^{d,e}$ ± 1.9	15.8^{b} ± 1.8	6.8^{e} ± 1.2	$8.4^{d,e}$ ± 0.6	$8.2^{d,e}$ ± 1.5

Table 7.8 Effect of fungal treatments on shikimates and other miscellaneous compounds in boronia marc over four days of incubation. The data, which is the ratio of the peak area of diagnostic ions to the peak area of the internal standard is presented as the means and standard errors of four replicates. T- test distributions are included as superscripts.

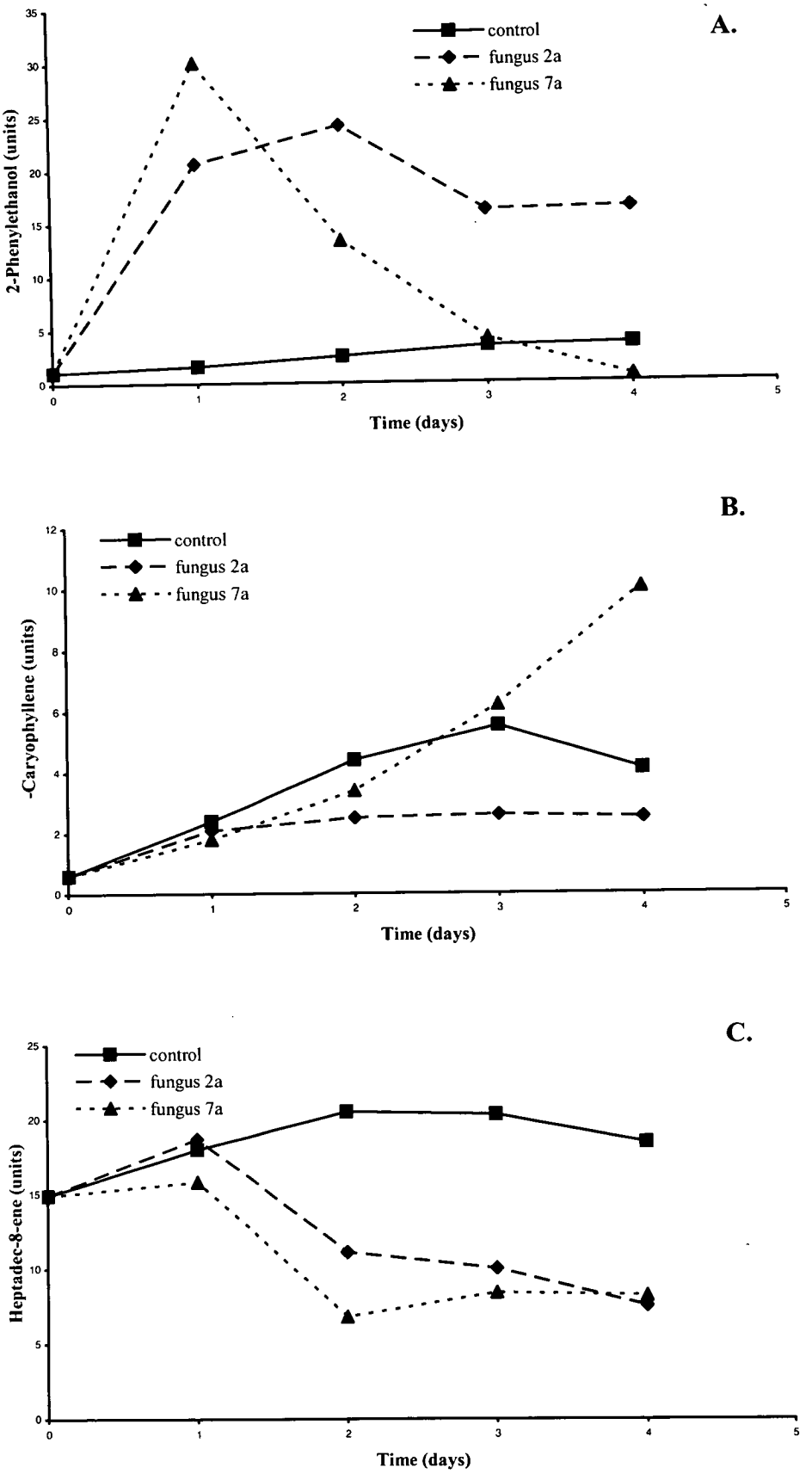


Figure 7.8 Relative changes in the levels of "other" compounds during fungal incubations. **A.** 2-Phenylethanol; **B.** β-Caryophyllene; **C.** Heptadec-8-ene. The results are expressed as arbitrary units which are the ratio of the peak area of target ions to the peak area of the internal standard.

al., 2004) was not observed in the blank or control incubations but was apparent in the extract after 3 days of treatment with fungus 2a.

Changes in the levels of two further alcohols, dodecanol and spathulenol, which are normally found in extracts from boronia were additionally measured during this experiment. Whilst the dodecanol level was observed to double during the control treatment the compound was observed to rapidly decrease due to both fungal treatments. Spathulenol also increased significantly during the control treatment and a small but significant increase was observed after 1 day of treatment with fungus 2a. No other significant differences were observed. Results for heptadec-8-ene (see **figure 7.8 C**) were similar in both treatments groups with no increases beyond that observed for the control incubations. The change in the levels of dodecyl acetate during control and fungal treatments indicated that both fungi were able to metabolise this compound.

In addition, it is important to consider the increase in the levels of the compounds in this category during the control incubations. Overall, the results indicated that increases in some compounds represented a doubling during the control incubation as was found for a variety of compounds in the other groupings. This is suggestive that the compounds were released from plant tissues during the incubation period resulting in increased availability to the extraction process. β-Caryophyllene (see **figure 7.8 B**) which is found in boronia at low levels (Weyerstahl *et al.*, 1995) also showed a significant increase during the control incubation. During the fungal treatments this compound showed the highest increase at day 4 of treatment with fungus 7a and the possibility of fungal biosynthesis should be considered.

Summary

The Time Trial experiment contributed further detail to the initial four day experiment and allows choices to be considered with regard to incubation with fungal isolates as a commercial tool for obtaining a further extract from the marc. With regard to that a number of key outcomes are detailed:

1. This experiment indicated that increases in volatiles were optimum after 1 – 2 days of fungal treatment.

2. Treatment of fungal isolates resulted in the metabolism of compounds that may have important aroma characteristics. These compounds include the cinnamic acid esters, linalyl esters, and tiglamides all of which elute on GC following octadecane.
3. Release of hydroxylated volatiles was apparent in several classes of compounds including the ionones, monoterpenols, shikimates, and cucurbates. The monoterpene alcohols released included monoterpenols and monoterpenediols as would have been expected on the basis of the monoterpene alcohols detected through the screening process.
4. Some newly released alcohols are metabolised at longer incubation times.
5. The release of hydroxy ionones appeared to be related to the formation of oxo derivatives of the released alcohols. This was especially apparent for the relationship between 3-hydroxy-5,6-dihydro-β-ionone and 3-oxo-5,6-dihydro-β-ionone in treatment with fungus 7a.
6. Volatiles normally found in boronia but which are unable to form glycosides increased during the control incubation.

The main issue associated with the possible use of these fungal isolates at a commercial level is the level of volatile release versus further metabolism of the released volatiles and other compounds in the marc. This issue is highlighted through the variable increases in volatiles due to the two different fungal treatments at any one time point. Overall, the changes are complex and substantial work remains especially with regard to determining the relative importance of volatiles, both newly released and un-extracted in the primary extraction, to the flavour and aroma of a potential new boronia extract.

Chapter 8

General Discussion

These studies have provided considerable insight into the possibility of increasing the yield of valuable aroma and flavour volatiles from boronia marc. The development of these insights was assisted through a body of chemical and physiological knowledge acquired over the past two decades of boronia research in Tasmania. From a chemical point of view there are three seminal studies. Davies and Menary (1983) presented a detailed examination of the composition of the Tasmanian boronia extract and provided the basis for further work by Weyerstahl *et al.* (1994) and Weyerstahl *et al.* (1995). The latter study is an invaluable resource which lists 129 components along with some mass spectrometric data and retention indices for each compound. Additionally, the Weyerstahl papers have assisted the Tasmanian research group in the compilation of a mass spectral library that facilitates identification of volatiles examined in research projects.

Physiologically, a series of research papers that followed from doctoral studies by MacTavish (1995) provided insights into both the potential of post harvest processes, and the direction for future research. A series of studies optimised the solvent extraction process (MacTavish and Menary, 1998b) and harvest time technologies (MacTavish and Menary, 1997). Experiments had also been conducted that explored research directions that are relevant to this study. Firstly, a series of experiments (MacTavish and Menary, 1999b and 1999c) led to the implementation of a commercial post harvest incubation process which resulted in increases in the yield of β-ionone (MacTavish and Menary, 2000). Secondly a range of very preliminary studies attempted to further understand the range of carotenoid (MacTavish, 1995) and glycosidic (MacTavish *et al.*, 2002) precursors present in boronia flowers, of aroma and flavour volatiles including C-13 norisoprenoids.

Based on this previous work, this study used innovative approaches to explore both the range of secondary metabolites relevant as either precursors or co-metabolites of aroma and flavour volatiles and possible post harvest technologies for the release of

these volatiles. Initial experiments, which aimed at understanding the *in vivo* production of C-13 norisoprenoids, focussed on attempting to detect a carotenoid cleavage enzyme in aqueous boronia extracts at a stage when, whilst it was considered highly probable, there was no direct evidence in the literature that β-ionone and other C-13 norisoprenoids were derived from C-40 carotenoids (Winterhalter and Rouseff, 2002). Whilst more recently, carotenoid cleavage dioxygenases have been isolated from star fruit (Fleischmann *et al.*, 2003) and nectarines (Baldermann *et al.*, 2005), the absence of progress with regard to cleavage enzymes in boronia led the project in a different direction.

Prior to the elucidation of carotenoid cleavage enzymes, the strongest evidence for the biogeneration of C-13 norisoprenoids from C-40 carotenoids was through the demonstrated presence of apocarotenoid fragments in plant extracts. This has been comprehensively discussed for starfruit (Winterhalter and Schreier, 1995), saffron (Winterhalter and Straubinger, 2000; Cadwallader, 2002), quince (Lutz and Winterhalter (1992) and roses (Eugster and Marki-Fischer (1991). The results in this study demonstrated the presence of a range of C-27 apocarotenoids including isomers of 3-hydroxy-apo-10′-carotenoic acid, methyl 3-hydroxy-apo-10′-carotenoate, apo-10′-carotenoic acid, apo-10′-carotenal, and methyl apo-10′-carotenoate (Cooper *et al.*, 2003). The carotenoic acids and their methyl esters had not previously been reported as occurring in plants.

A consideration of the structures of these compounds helped to build a picture of the metabolic processes occurring in boronia flowers which in turn allowed insight into possible biotechnological processes for increasing yields from the flowers. Related to this, consideration of the structures of the newly discovered apocarotenoids and the known C-13 norisoprenoids present in boronia extracts (Weyerstahl *et al.*, 1995) provided strong evidence that both hydrocarbon carotenoids and xanthophylls are cleaved to produce β-ionone and hydroxy ionones. In addition, the presence of methyl esters of the apocarotenoids demonstrated methyl transferase activity in the flowers which is an important biochemical function with regard to the biochemistry of plant volatiles (Dudereva *et al.*, 2004). Methyl esters of volatiles compounds found in the boronia extract include methyl cinnamates, jasmonates and cucurbates (Weyerstahl *et al.*, 1995).

Changes in the levels of the newly discovered C-27 apocarotenoids, β-ionone and their C-40 carotenoid precursors were measured during flower development. These changes, with minor exceptions, were consistent with the observations by MacTavish (1995) that β-ionone levels were first apparent at the large bud stage of flower development. The results indicated that there was a turning on of carotenoid biosynthesis and cleavage beginning just prior to bud opening. Previously, the research of MacTavish (1995) had focussed on β-ionone. This was primarily because of the high levels of this compound in the extract and its important flavour and aroma characteristics that have been widely recognised by the food and perfumery industries (Arctander, 1960; Mookherjee *et al.*, 1995). It was timely then, given that xanthophyll cleavage in the 9,10 position was indicated, to consider the possible metabolic processes with regard to the formation of glycosidic conjugates in boronia.

An HPLC / MS screening process was used successfully to detect compounds in the marc extract that were consistent with the glycosides of known volatiles. The proposed identities of simple (mono-carbohydrate) and malonyl glycosides of C-13 norisoprenoids, monoterpenols, and cucurbates were consistent with the results from selective treatment with β-D-glucosidase and pectinase enzyme preparations. The overall outcomes from the enzyme treatments were that:

1. The putative malonyl glycosides were hydrolysed by the AR 2000 pectinase treatment and not by the β-D-glucosidase treatment. The results provided strong additional evidence to support the hypothesis that malonyl glycosides were present in boronia, as pectinases are prepared from fungal sources and are known to contain esterases which are capable of hydrolysing malonyl glycosides in combination with glycosidase activity (Roscher *et al.*, 1997).
2. The simple glycosides were hydrolysed by both the pectinase and β-D-glucosidase treatments. This is consistent with the simple glycosides being present as glucosides. A qualitative determination that a hexyl carbohydrate was released in these trials was also consistent with the proposed general identity (unpublished observation).
3. The volatiles released through the enzyme treatments confirmed that C-13 norisoprenoids, monoterpenes and cucurbates were the main volatiles released from the mixture. These results showed much higher release levels

than were reported by MacTavish et al. (2002) and the absence of β-ionone which was reported by that author as increasing following treatment with endogenous enzyme extracts.

Evidence for the presence of malonyl glycosides in boronia is a significant finding as, although they have been commonly reported for polyphenolic compounds (Kudou *et al*, 1991; Grün *et al.*, 2001), there have been limited reports on the conjugation of flavour and aroma compounds as malonylated glycosides (Schwab and Schreier, 1988; Moon *et al.*, 1994; Roscher *et al.*, 1996; Watanabe, 1997). A more wide ranging study (Withopf *et al.*,1997) found these compounds in mountain papaya, the leaves of green tea and grape vines, and various fruits (guava, raspberry, strawberry).

The model described here, which utilised an Atmospheric Pressure Chemical Ionisation (APCI) HPLC / MS screening process in combination with enzyme hydrolysis, was an effective screening process which yielded valuable information with regard to the conjugate profile of boronia. This screening technique could be applied to other plant products and / or experimental problems. However prior purification and spectroscopic identification of individual glycosides or the use of chemically synthesised reference standards as was used by Withopf *et al.* (1997) and Boss *et al.* (1998) would provide more definitive information. Those authors, belonging to the same research group, screened for glycosides in several different types of fruits and leaves using Electrospray Ionisation (ESI) MS / MS with the analysis performed on a triple stage quadropole MS / MS analyser. However, they had detailed the use of APCI, as was used in this study, in an earlier report (Roscher *et al.*, 1996).

This combination of techniques allowed the development of insights and a series of further experiments that would not have been possible if the conjugates had only been detected through hydrolysis studies. The experimental approach developed here was firstly the use of a screening technique followed by a differential enzymatic exploration of the general identities of the conjugate groupings. The success of this approach allowed the relatively simple methodology developed in the screening process to monitor changes during trials of purification techniques and the

examination of differences including in clones and at stages of flower development. In contrast, Bureau *et al.* (2000b) used only enzymatic hydrolysis with a pectinase to measure the effect of vine and bunch shading on grape berry glycosides.

Although the methodology used did not allow characterisation of individual glycosides, by considering both the volatiles released and the effects of the enzyme treatments on the various conjugate groupings, several conclusions were possible with regard to glycoside forms. Linalool is the only (mono – hydroxy) monoterpenol detected as a released volatile and because no simple glycosides of monoterpenols were detected in the screening process then linalool must be present as a putative malonyl glycoside. Previously, linalyl 6'-malonyl-β-D-glucopyranoside was detected in the flowers of *G. jasminoides* (Watanabe, 1997). It is possible that the monoterpenediols are present as both simple glycosides and malonyl glycosides. A similar logic applies to the C-13 norisoprenoids although it appeared that the cucurbates were only apparent in the malonyl glycosidic grouping.

Whilst the screening process included a search for ionols and for different forms of glycosides including disaccharides, it is still possible that other glycosidic conjugate groups are yet to be detected in boronia. It was however notable that β-ionols were absent from the group of released C-13 norisoprenoids. This is in contrast to other plant extracts including from lulo leaves (Osorio *et al.*, 1999), apple leaves (Stingl *et al.*, 2002) and raspberry fruits (Pabst *et al.*, 1991) where both ionol and hydroxy ionone glycosides have been either isolated or detected following enzymatic hydrolysis of extracts. Also notable was the release of 4-hydroxy-β-ionone from the extract following enzymatic treatment. Winterhalter and Schreier (1994) reported that 4-oxygenated glycosides of C-13 norisoprenoids occur only rarely in nature and referred to raspberry fruit as an example. More recently glycosidically bound 4-hydroxy-β-ionone has been detected in Mammee Apple fruit pulp (Duque *et al.*, 2002).

Winterhalter and Schreier (1994) made no comment as to whether their observation might be due to the relative unavailability of 4-hydroxy ionones linked to the occurrence of carotenoid precursors. A survey of the carotenoids described by Straub (1987) indicated that hydroxylation in the four position is relatively rare. No 4-

hydroxy carotenoids have been identified in boronia but the occurrence of 4-hydroxy-β-ionone in both the free and bound forms prescribes the need for further identification of unknown carotenoids in boronia. With regard to the structural elucidation of glycosidic conjugates, although NMR spectroscopy is the definitive methodology for structural determination, other authors have used enzymatic liberation to confirm the presence of glycosidic conjugates. However, it would be beneficial as part of a program of future research to elucidate the structures of the glycosidic conjugates. In general the identification of precursor compounds will enhance the capacity of industry and researchers to make good decisions about future strategies related to both clonal development and post harvest processing techniques.

The experiments conducted in this study have allowed the development of a proposal for the future isolation and purification of individual conjugates. **Figure 8.1** outlines a proposal for this further purification. This separation scheme differs from literature methodologies through the use of Sephadex G-10 chromatography which is proposed for the removal of polyphenols. Aqueous plant extracts are commonly high in polyphenolic compounds and boronia was observed to contain high levels of rutin and related methyl derivatives. The presence of rutin in boronia created difficulties with regard to the MS / MS screening process. In data dependent mode the largest ion peak, automatically selected for the generation of MS^2 spectra, was in some cases derived from rutin which dominated a significant elution time window. Strategies including G10 chromatography were developed to remove polyphenols from the boronia extract. One of the more usual methods for removing polyphenolic compounds is the use of polyvinylpolypyrrolidone (PVPP) (Doner *et al.*, 1993). However, preliminary trials conducted with PVPP were equivocal with regard to the effectiveness of this as a pre-treatment and there was some evidence that malonyl glycosides bind to the resin. It may be important to resolve this issue as this method has been previously applied to the preparation of glycosidic extracts (Pabst *et al.*, 1992; Wang *et al.*, 2000).

Without the availability at the University of Tasmania of any of the forms of high speed countercurrent chromatography that have been proved useful for the separation of glycosidic conjugates by other researchers (Osorio *et al.*, 1999; Straubinger *et al.*,

1997; Humpf and Schreier, 1992; De Tommasi *et al.*, 1996) pre-fractionation using column chromatography with RP-C18 Wang *et al.* (1998) of the polyphenol free

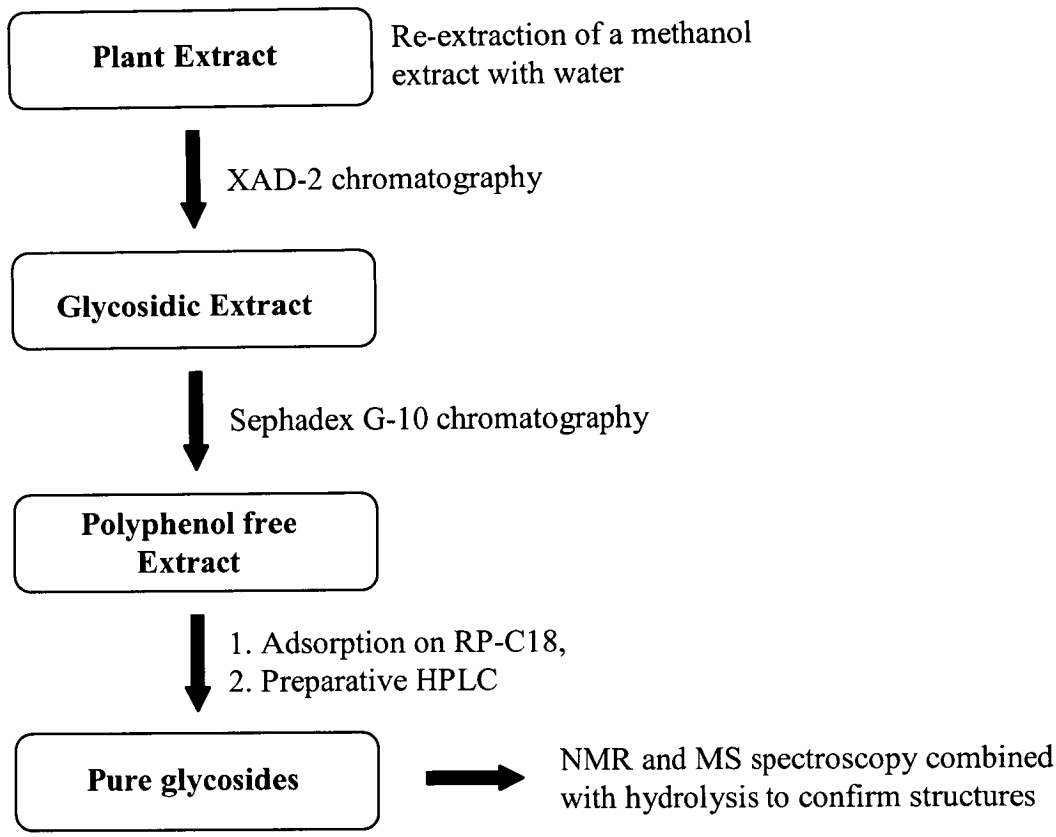


Figure 8.1 Proposed scheme for purification and identification of individual glycosidic conjugates.

extract followed by preparative HPLC (Straubinger *et al.*, 1997) would seem an appropriate separative pathway. Additionally, in future studies some consideration could be given to the separation of malonyl glycosides from simple glycosides through solid phase extraction based on their different absorptive capacities. An anion exchange process, that utilised the carboxylic acid functional group on the malonyl glycoside, might possibly effect a separation between malonyl glycosides and other simple glycosides in the polyphenol free extract.

Following confirmation of the presence of glycosidic conjugates of hydroxy ionones monoterpenes and cucurbates, clonal variation and differences during flower development were explored. The changes in glycoside levels during flower development showed a similar pattern of increase to that observed for C-27 apo-carotenoids and β-carotene with levels increasing throughout flower development. It was useful here to make some comparisons to the work of Watanabe (1997) who

examined the changes in glycosidic precursors during flower development in *Gardenia jasminoides*. Watanabe examined *G. jasminoides* flowers at similar stages of flower development to those selected for this study presenting results for buds at different stages of development, flowers just after opening, and open flowers. Precursor changes were examined through hydrolysis of the conjugates with different enzyme systems. As was observed in boronia, Watanabe was able to show that precursors were present at the lowest levels in immature buds with levels increasing around the time of flower opening.

Variation between the conjugate levels in the commercially available boronia clones (3, 250, 5 and 17) did not show any consistent trends. However clone 3 flowers contained the highest levels of the putative malonyl glycosides of C-13 norisoprenoids and cucurbates. Although previous post harvest studies with boronia relate to a different process it should be noted that MacTavish and Menary (1999b) studied differences between clones during a post harvest incubation (prior to the primary extraction) and observed that clone 250 showed the highest increase in β-ionone and the yield of total volatiles. In these studies clone 250 marc was observed to have the highest levels of the proposed malonyl glycosides of monoterpenols (assumedly linalool – see prior discussion). Presumably changes in the post harvest incubations of MacTavish and Menary (1999b) relate to *in vivo* enzymatic cleavage of carotenoids whilst the changes observed here are through the metabolic action of fungi that are naturally occurring in association with boronia. There is no evidence that the post harvest process observed by MacTavish and Menary (1999b) occurred through the action of fungi especially with regard to the overall low accumulation of carotenoids in boronia observed in this study.

Further studies compared the levels of conjugates in flowers and buds with their subtending leaves. These results showed major differences between the leaves and flowers with the subtending leaves of flowers and buds showing high levels of similarity. The malonyl glycosides of C-13 norisoprenoids showed a different pattern of appearance between leaves and flowers with the absence of individual glycoside peaks in some cases. This contrasted with studies on wines where it was concluded that C-13 norisoprenoid conjugate levels were higher in leaves than in berries (Günata *et al.*, 2002)

Following on from the studies on conjugate levels in flowers and leaves a series of incubation trials using clone 3 flowers led to the development of a potential commercial process that resulted in the release of aroma and flavour volatiles with a concurrent 36% increase in the yield of extract from boronia. Importantly the new extract was 87% absolute which compared favourably with the primary extract (from fresh flowers) which contains about 50% absolute. This simple marc incubation process was further explored through the examination of the effect of endogenous fungi, which had been isolated from areas of obvious microbiological growth during the incubation process, on sterilised marc. Fungal isolates that were effective in hydrolysing boronia glycosides were identified as *Penicillium* and *Aspergillus* species. *Aspergillus niger* has been widely recognised as containing glycosidase activity and the enzymes have been extracted for commercial purposes (Spagna *et al.*, 1998; Spagna *et al.*, 2000; Wei *et al.*, 2004).

These experiments allowed some important principles to be established. Firstly, both the simple incubation process and incubation with fungal isolates showed the release of flavour and aroma volatiles including important monoterpenols and C-13 norisoprenoids. Secondly the incubation process led to an extract that improved the overall yield of concrete from boronia. Thirdly it was observed through organoleptic assessment that the new extracts contained aroma sensations that would add positive value to the currently available commercial extract. However, whilst these results may provide considerable benefit to the boronia industry there is need for caution and refinement of the developing technology. This is based on the following observations. Firstly, organoleptic assessment of the product obtained during the pilot scale commercial trials resulted in the observation that the linalyl esters, tiglamides and cinnamates, which elute on GC following octadecane and are presumably inefficiently extracted in the primary commercial flower extraction process, may contribute important aroma sensations to the extract. It was further observed that these compounds underwent metabolism during the incubation period. Further studies will need to properly assess the aroma value of these groups of compounds. Appropriate strategies may include the synthesis of reference standards.

Other issues that need to be considered include the further metabolism of released volatiles and the possibility of aroma enhancement of released monoterpenediols through acid treatment. These considerations might lead to an alternative commercial process or to refinement of a fungal incubation technique that maximises the process of volatile generation and minimises any adverse effects. Refinement of the fungal incubation technology may be possible through manipulation of water activity, temperature and pH (Gibson and Hocking, 1997). The initial step would be to measure the optimum growth factors of fungal isolates and then apply that knowledge to pilot or commercial-scale trials. With regard to the simple marc incubation it might be possible to maximise the release of flavour and aroma compounds from precursors whilst preventing unfavourable metabolic effects of other fungi or bacteria in the simple marc incubation technique by inhibiting their growth. In work with fungal isolates alone it may be possible to reduce the time and increase the efficiency of the incubation.

Whilst a potential commercial process has been developed, which profoundly increased the yields of volatiles and extract from boronia marc and has further potential for refinement, perhaps the greatest strength of the study then is that it lays a foundation for future research and protocol development. Recommendations for the future include a series of commercial scale comparative studies along with development of an analytical strategy for the selection of future clones. A series of recommendations for future commercial trials are presented which are in addition to the fungal incubation technology. They are based both on the experiments reported in this study and literature knowledge. The aim of these future studies should be to develop a commercial process which further balances the overall yield and the aroma and flavour quality of that extract. Future trials should include:

1. Simultaneous Distillation-Extraction (SDE)

Simultaneous Distillation-Extraction has been applied to numerous plant extracts. With passionfruit Engel and Tressl (1983) used an SDE model with thermal treatment and without pH modification. Diluted passionfruit pulp has a "native" pH of 3.0 and the yield of volatiles was considerably reduced when the pH was adjusted to 7.0. Using model experiments the authors were able to demonstrate that monoterpenols can be derived from monoterpenediols. These compounds were

described as non-volatile precursors although it has been noted by other authors that some monoterpenediols have flavour and aroma characteristics and 8-hydroxy linalool was reported by Weyerstahl et al. (1995) as having fatty, green herbaceous, woody, and citronella-like aroma sensations. The two monoterpenediols released following enzyme and fungal incubation experiments were 7-hydroxy hotrienol (3,7-dimethylocta-1,5-diene-3,7-diol) and 8-hydroxy linalool (3,7-dimethylocta-1,6-diene-3,8-diol). The former compound is important because it has been widely recognised as an odourless precursor to hotrienol which is formed in acidic conditions (Wintoch *et al.*, 1993). It is possible that a related extraction process using an appropriate pH might improve the quality of the extract through conversion of the monoterpenediols to compounds with improved aroma characteristics.

2. Commercial Scale Pectinase Trials.

The use of pectinase enzymes for processing the marc should be explored at a commercial level with such use eventually dependent on the overall cost of enzyme treatment in comparison to the simple marc incubation and / or treatment with fungal isolates. The key reason for exploring this process at a commercial level is that the process has the potential to release all of the glycosidic conjugates without further metabolism of flavour and aroma compounds that were not extracted in the primary commercial extraction of flowers. This will need to be balanced against a detailed assessment of the aroma qualities of the extracts from different treatments.

The effects of the various enzyme and fungal treatments explored in this study on secondary metabolites related to linalool is worthy of more detailed exploration with regard to the *de novo* metabolic processes in boronia. Effects on linalool, 8-hydroxy linalool, 8-hydroxy linalool esters and a newly discovered unknown linalool related metabolite were observed and the discussion is important for two reasons. Firstly monoterpenols are important flavour and aroma compounds in many plant extracts (Flament, 1991; Latrasse, 1991). Related to this linalool has been described as having a floral aroma sensation (Latrasse, 1991) which would be compatible with the boronia extract. Secondly, there are 21 linalool related compounds listed by Weyerstahl *et al.* (1995) as having been identified in the extract.

Linalool and 8-hydroxy linalool were both demonstrated to be released through treatment with β-D-glucosidase and the pectinase preparation. It is proposed here that a hydroxylation pathway from linalool to 8-hydroxy linalool exists in boronia flowers and this allows some speculation with regard to a linalool related cluster of metabolites. **Figure 8.2** is a simple schematic profile proposing metabolic pathways that lead to the formation of these esters from precursors that have been found to occur in boronia (Weyerstahl *et al.*, 1995).

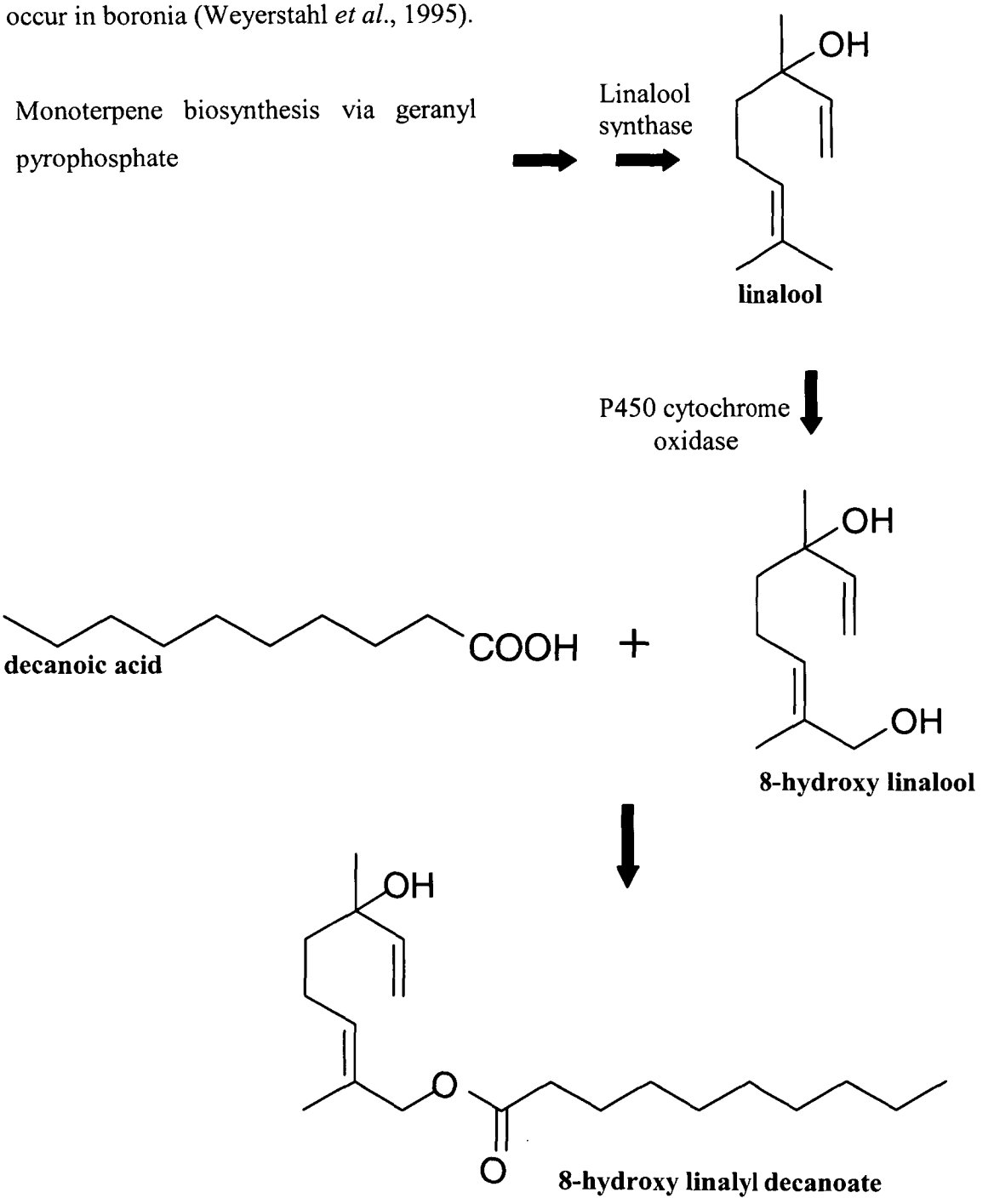


Figure 8.1 Biosynthetic pathway showing the likely route to 8-hydroxy linalyl esters in *Boronia megastigma*. Whilst the example shown here is specific for 8-hydroxy linalyl decanoate, numerous 8-hydroxy linalyl esters have been found in boronia that might be formed through this process.

The range of 8-hydroxy linalyl esters in boronia is extensive and may imply a high level of linalool biosynthesis. A comprehensive review of linalool biosynthesis in relation to biometabolism in *Clarkia breweri* (Raguso and Pichersky, 1999) indicated that linalool is a precursor of other structurally related aroma molecules including pyranoid and furanoid linalool oxides. Those compounds have not been detected in boronia (Weyerstahl *et al.*, 1995) but it is highly probable that extensive metabolism in relation to linalool is occurring within boronia especially with regard to conversion to di-oxygenated monoterpenols and esters. A more thorough exploration of this metabolism may aid the development of processes that take advantage of these metabolic pathways with regard to further improving monoterpenol yields in boronia.

Relating to this, there are some key considerations with regard to future biotechnological manipulation of boronia that takes advantage of this knowledge. Firstly, given that the post C-18 compounds may contribute favourably to the aroma profile of boronia it is necessary to consider in which form the linalool related compounds will contribute most favourably to an increase in extract quality. Secondly, more detailed clarity is necessary with regard to both identification of the unknown linalool related compound and insight into the breakdown pathways induced by either pectinase preparations or fungal treatments. With regard to the latter point the potential of linalyl ester cleavage to lead to monoterpenol accumulation in treated marc needs to be investigated. In this study the simple marc incubation and treatments with fungal isolates led to the disappearance of some linalyl esters. However the linalyl esters are potential aroma compounds as evidenced by the GC / Olfactometry studies of marc extract. Additionally the possibility exists that the unknown linalool related compound is derived from these esters and the aroma potential of this unknown compound and the esters is yet to be determined.

A consideration of linalool metabolism has been used to develop an insight into the discussion around the site of biosynthesis of glycosidic conjugates. Labelling studies with ^{18}O-labeled (R/S)-linalool (Burkhardt and Mosandl, 2003) and deuterium labelled linalool related precursors (Kreck *et al.*, 2003) explored the biosynthesis of lilac aldehyde stereoisomers from linalool. These studies provide evidence for *de*

novo biosynthesis of monoterpenols in the flowers in apparent contradiction to the leaf to flower transport theory attributed to Watanabe *et al* (1993) which suggested biosynthesis and glycosylation of monoterpenols in leaves prior to transport to and release in flowers. Given that un-hydroxylated plant volatiles including monoterpenes (which cannot be glycosylated) occur in flowers, it is likely that biosynthesis of these volatiles occurs in the flowers directly. The finding of C-40 carotenoid cleavage products, especially hydroxy C-27 apocarotenoids, during flower development in boronia during this study is additional evidence for *de novo* biosynthesis of hydroxylated volatiles in flowers. Furthermore, the absence of, or occurrence of different pattern of appearance, between flowers and subtending leaves is also supportive of conjugate biosynthesis in boronia flowers. The argument against biosynthesis of monoterpenols in leaves is also supported in studies on *Clarkia breweri* where an "obligate precursor relationship between linalyl glycosides and free linalool emissions" could not be demonstrated (Raguso and Pichersky, 1999). However, Gholami *et al.* (1996) reported the presence of glycosides in grape petioles which might be considered evidence for leaf flower transport.

The evolution in the literature of the rationale that volatiles are synthesised in the leaves and transported to the flowers is complex and involves several inter-related reports. Winterhalter and Skouroumounis (1997), in a review of glycoconjugated aroma compounds, stated that this mechanism was suggested by Watanabe *et al* (1993). A careful examination of the later paper found that the authors referred to Ackermann *et al* (1989) as the source for the leaf – flower transport rationale. Prior to that Croteau (1987), who elegantly demonstrated the importance of glycosylation for the transport of monoterpene volatiles in *Mentha arvensis*, referred to two studies where "terpenyl glycosides had been transported from leaves to flowers in rose". However, a careful reading of those reports (Bugorskii *et al.*, 1979; Pogorel'skaya *et al.*, 1979) indicated that whilst referring to the concept on the basis that glycosides can be synthesised in the leaves did not present evidence for leaf to flower transport. Furthermore, Pogorel'skaya (1979) reported that the radioactive label from C14 sucrose taken up into flowers via the stem was incorporated into essential oil components, thus indicating the presence in the flower of biosynthetic pathways for essential oil production from carbohydrates. Overall then, there is very little

evidence in the literature for leaf to flower transport. Studies with boronia support the rationale that biosynthesis of glycosides of volatiles occurs in the flowers.

Overall it is clear that an understanding of the biochemistry and physiology of boronia has led to an increased understanding of the capacity to increase the yields of valuable products from the marc. The use of waste material from agricultural products that have been extracted or utilised to obtain a food product is becoming more common as advances in technology allow this use to become more economically viable. Given this increasing ability to obtain a higher range of valuable products from a commercial crop it is in turn important to consider issues of environmental sustainability. The idea that technological gains can be traded for a more sustainable use of agricultural land has been advanced by Hawken *et al.* (1999). This thesis contains a concrete example of how technology can be used to make substantial productivity gains.

In addition to higher yields of flavour and aroma compounds, boronia has the potential to offer yields of other high value extracts such as anthocyanins. Boronia is considered to be high in anthocyanins and preliminary assessments of the potential for anthocyanin extraction from boronia have been conducted (personal communication, R. Menary). Furthermore, these studies indicated that boronia has high levels of anthocyanins and other polyphenolic compounds. This was evidenced by the HPLC chromatography of methanolic and aqueous extracts (unpublished data, this laboratory). In the experiments conducted for this study the anthocyanin colouration was observed to disappear with both pectinase and fungal treatments. The presence of enzymes in fungal enzyme extracts, with the ability to hydrolyse anthocyanins leading to decolourisation, has been known since the 1950's (Huang, 1956). In relation to this Wightman *et al.* (1997) found that commercial pectinases, including AR 2000, were able to catalyse the hydrolysis of anthocyanins in wine leading to some decolourisation. A commercial resin was found by Scordino *et al.* (2005) to aid the recovery of anthocyanins and hydroxycinnamates from citrus pulp thus providing the potential for a high value product with several commercial applications. Such an application has relevance with regard to boronia technologies. Therefore, it is clear that the use of new technologies for the production of volatiles

in boronia might need to be considered with regard to the potential for using boronia marc as a source of anthocyanins.

In summary, this study has provided key information relating to aspects of volatile biosynthesis and product extraction as outlined in the schematic representation of study areas referred to in the literature review (**figure 1.6**). The discovery of a series of C-27 apo-carotenoids provided important evidence to support the hypothesis that C-13 norisoprenoids were derived from C-40 carotenoids through site specific cleavage. This rationale was also supported by the concurrent appearance of β-ionone and C-27 apocarotenoids during flower opening. A methodology using HPLC / MS was developed and used to screen for glycosidic conjugates in boronia. The presence of both simple glycosides and malonyl glycosides of C-13 norisoprenoids, monoterpenols and cucurbates were proposed and their general identities indicated through hydrolysis with a fungal pectinase (AR 2000) and β-D-glucosidase. These conjugates were demonstrated to appear during flower development.

A simple incubation process was developed through to a pilot commercial scale that resulted in an increased yield of flavour and aroma compounds. Incubation of sterilised marc with fungal isolates obtained from the flowers during the simple incubation process resulted in the release of flavour and aroma compounds. The incubation experiments provided additional insights. In addition to the glycosidic precursors there are a large range of other compounds not extracted efficiently in the primary flower extraction process. These compounds include un-extracted volatiles and larger molecular weight compounds (eg. α-tocopheryl and β-amyrin esters) not usually detected in the conventional GC analyses. Several tiglamides, linalyl esters and cinnamate esters were demonstrated using GC/ Olfactometry to have aroma value. These compounds were also metabolised during the incubation processes.

This work provided important insight into the biochemistry associated with release of flavour and aroma compounds from carotenoid and glycosidic precursors. The potential for an increased yield of a valuable extract from the waste flowers is clearly demonstrated and the study provides clear guidelines for future work which will maximise both the economic value of the potential new products from boronia and related scientific knowledge.

Bibliography

Ackermann, I. E., Banthorpe, D. V., Fordham, W. D., Kinder, P., and Poots, I. (1989). β-Glucosides of aroma components from petals of Rose species: Assay, occurrence, and biosynthetic implications. *Journal of Plant Physiology, **134***, 567-572.

Al Hasani, S. M., and Parrish, D. B. (1972). Forms of vitamin A and of carotenoids in tissues, blood serum, and yolk of eggs from *Coturnix coturnix japonica* fed apo-β-carotenals. *Journal of Nutrition, **102***, 1437-1440.

Angelino, S. A. G. F. (1991). Beer. In H. Maarse (Ed.), *Volatile Compounds in Foods and Beverages* (pp. 581-616). New York: Marcel Dekker, Inc.

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin*. Elizabeth: Arctander, S.

Aubert, C., Ambid, C., Baumes, R. L., and Günata, Z. (2003). Investigation of bound aroma constituents of yellow-fleshed nectarines (*Prunus persica* L. Cv. Springbright). Changes in bound aroma profile during maturation. *Journal of Agricultural and Food Chemistry, **51***, 6280-6286.

Baek, H. H., and Cadwallader, K. R. (1999). Contribution of free and glycosidically bound volatile compounds to the aroma of Muscadine grape juice. *Journal of Food Science, **64***, 441-444.

Baldermann, S., Naim, M., and Fleischmann, P. (2005). Enzymatic carotenoid degradation and aroma formation in nectarines (*Prunus persica*). *Food Research International, **38***, 833-836.

Bartley, G. E., and Scolnik, P. A. (1995). Plant carotenoids: Pigments for photoprotection, visual attraction, and human health. *The Plant Cell, 7*, 1027-1038.

Baxter, R. L., Laurie, W. A., and McHale, D. (1978). Transformation of monoterpenoids in aqueous acids. The reactions of linalool, geraniol, nerol and their acetates in aqueous citric acid. *Tetrahedron, **34***, 2195-2199.

Bazemore, R., Rouseff, R., and Naim, M. (2003). Linalool in orange juice: Origin and thermal stability. *Journal of Agricultural and Food Chemistry, **51***, 196-199.

Belancic, A., Günata, Z., Vallier, M.-J., and Agosin, E. (2003). β-Glucosidase from the grape native yeast *Debramyces vanrijiae*: Purification, characterisation, and its effect on monoterpene content of a muscat grape juice. *Journal of Agricultural and Food Chemistry, **51***, 1453-1459.

Bertolini, A. C., Siani, A. C., and Grosso, C. R. F. (2001). Stability of monoterpenes encapsulated in gum arabic by spray-drying. *Journal of Agricultural and Food Chemistry, **49***, 780-785.

Bicalho, B., Pereira, A. S., Aquino Neto, F. R., Pinto, A. C., and Rezende, C. M. (2000). Application of High Temperature Gas Chromatography - Mass Spectrometry to the investigation of glycosidically bound components related to cashew apple (*Anacardium occidentale* L. Var. *nanum*) volatiles. *Journal of Agricultural and Food Chemistry, **48***, 1167 - 1174.

Biswal, B. (1995). New trends in photobiology (invited review): Carotenoid catabolism during leaf senescence and its control by light. *Journal of Photochemistry and Photobiology B: Biology, **30***, 3-13.

Boss, B., Richling, E., and Schreier, P. (1998). HPLC-ESI-MS/MS analysis of 6'-O-malonylated β-D-glucosides in plants. In P. Schreier, M. Herderich, H.-U. Humpf & W. Schwab (Eds.), *Natural products analysis. Chromatography, spectroscopy, biological testing.* (pp. 187-191). Wiesbaden: Wieweg.

Bosser, A., and Belin, J.-M. (1994). Synthesis of β-ionone in an aldehyde / xanthine oxidase / β-carotene system involving free radical formation. *Biotechnology Progress, **10***, 129-133.

Bosser, A., Paplorey, E., and Belin, J.-M. (1995). A simple way to (+/-)-dihydroactinidiolide from β-ionone related to the enzymic co-oxidation of β-carotene in aqueous solution. *Biotechnology Progress, **11***, 689-692.

Boulanger, R., Chassagne, D., Montesinos, M., Sakho, M., and Crouzet, J. (2000). The aromatic potential of Amazonian fruits. In P. Schieberle & K.-H. Engel (Eds.), *Frontiers of Flavor Science.* Freising: Deutsche Forschungsanstalt fur Lebensmittelchemie, Garching.

Bouvier, F., Dogbo, O., and Camara, B. (2003a). Biosynthesis of the food and cosmetic plant pigment bixin (Annatto). *Science, **300***, 2089-2091.

Bouvier, F., Suire, C., Mutterer, J., and Camara, B. (2003b). Oxidative remodelling of chromoplast carotenoids: Identification of the carotenoid dioxygenase CsCCD and CsZCD. *The Plant Cell, **15***, 47-62.

Bramley, P. M. (2002). Regulation of carotenoid formation during tomato fruit ripening and development. *Journal of Experimental Botany, **53***, 2107-2113.

Breithaupt, D. E., and Bamedi, A. (2001). Carotenoid ester in vegetables and fruits: A screening with emphasis on β-cryptoxanthin esters. *Journal of Agricultural and Food Chemistry, **49***, 2064-2070.

Breithaupt, D. E., and Bamedi, A. (2002). Carotenoids and carotenoid esters in potatoes (*Solanum tuberosum* L.): New insights into an ancient vegetable. *Journal of Agricultural and Food Chemistry, **50***, 7175-7181.

Brenna, E., Fuganti, C., and Serra, S. (2005). Synthesis and olfactory evaluation of the enantiomerically enriched forms of 7,11-epoxymegastigma-5(6)-en-9-one and 7,11-epoxymegastigma-5(6)-en-9-ols isomers, identified in *Passiflora edulis*. *Tetrahedron: Asymmetry, **16***, 1699-1704.

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *The FASEB Journal, **9***, 1551-1558.

Britton, G. (1998). Overview of carotenoid biosynthesis. In G. Britton, S. Liaaen-Jensen & H. Pfander (Eds.), *Carotenoids. Volume 3: Biosynthesis and Metabolism* (Vol. 3, pp. 13-147). Basel: Birkhauser-Verlag.

Britton, G., Liaaen-Jensen, S., and Pfander, H. (2003). *Carotenoids Handbook.* Basel: Birkhauser Verlag.

Buettner, A., and Welle, F. (2004). Intra-oral detection of potent odorants using a modified stir-bar sorptive extraction system in combination with HRGC-O, known as the buccal odour screening system (BOSS). *Flavour and Fragrance Journal, **19***, 505-514.

Bugorskii, P. S., Reznikova, S. A., and Zaprometov, M. N. (1979). Interrelationship between glucoside-bound and free alcohols during essential oil formation in rose petals. *Biokhimiya, **44***, 1068-1073.

Buonaurio, R., and Servili, M. (1999). Involvement of lipoxygenase, lipoxygenase pathway volatiles, and lipid peroxidation during the hypersensitive reaction of pepper leaves to *Xanthomonas campestris* pv. *vesicatoria. Physiological and Molecular Plant Pathology, **54***, 155-169.

Bureau, S. M., Baumes, R. L., and Razungles, A. J. (2000b). Effects of vine or bunch shading on the glycosylated flavor precursors in grapes of *Vitis vinifera* L. Cv. Syrah. *Journal of Agricultural and Food Chemistry, **48***, 1290-1297.

Bureau, S. M., Razungles, A. J., and Baumes, R. L. (2000a). The aroma of Muscat of Frontignan grapes: effect of the light environment of vine or bunch on volatiles and glycoconjugates. *Journal of the Science of Food and Agriculture, **80***, 2012-2020.

Burkhardt, D., and Mosandl, A. (2003). Biogenetic studies in *Syringa vulgaris* L.: Bioconversion of $^{18}O(^{2}H)$-labeled precursors into lilac aldehydes and lilac alcohols. *Journal of Agricultural and Food Chemistry, **51***, 7391-7395.

Bussell, B. M., Considine, J. A., and Spadek, Z. E. (1995). Flower and volatile oil ontogeny in *Boronia megastigma. Annals of Botany, **76***, 457-463.

Cabaroglu, T., Selli, S., Canbas, A., Lepoutre, J.-P., and Günata, Y. Z. (2003). Wine flavour enhancement through the use of exogenous fungal glycosidases. *Enzyme and Microbial Technology, **33***, 581-587.

Cadwallader, K. R. (2002). Flavor chemistry of Saffron. In P. Winterhalter & R. L. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 220-239). Washington DC: American Chemical Society.

Camara, B., and Bouvier, F. (2004). Oxidative remodelling of plastid carotenoids. *Archives of Biochemistry and Biophysics, **430***, 16-21.

Chassagne, D., Boulanger, R., and Crouzet, J. (1999). Enzymatic hydrolysis of edible *Passiflora* fruit glycosides. *Food Chemistry, **66**,* 281-288.

Chaudhuri, K., and Chawla, H. M. (1987). Anthroquinones and terpenoids from *Cassia javanica* leaves. *Journal of Natural Products, **50**,* 1183-1183.

Cheetham, P. S. (1997). Combining the technical push and the business pull for natural flavours. In R. G. Berger (Ed.), *Biotechnology of Aroma Compounds* (Vol. 55, pp. 1-50). Berlin: Springer-Verlag.

Chernys, J. T., and Zeevaart, J. A. D. (2000). Characterisation of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiology, **124**,* 343-353.

Cooper, C. M., Davies, N. W., and Menary, R. C. (2003). C-27 Apocarotenoids in the flowers of *Boronia megastigma* (Nees). *Journal of Agricultural and Food Chemistry, **51**,* 2384-2389.

Cortés, C., Esteve, M. J., Frígola, A., and Torregrosa, F. (2004). Identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with ultraviolet-diode array detection. *Journal of Agricultural and Food Chemistry, **52**,* 2203-2212.

Cox, A., Capone, D. L., Elsey, G. M., Perkins, M. V., and Sefton, M. A. (2005). Quantitative analysis, occurrence, and stability of (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene in wine. *Journal of Agricultural and Food Chemistry, **53**,* 3584-3591.

Croteau, R. (1987). Biosynthesis and catabolism of monoterpenoids. *Chemical Reviews, **87**,* 929-954.

Crouzet, J., and Chassagne, D. (1999). Glycosidically bound volatiles in plants. In R. Ikan (Ed.), *Naturally Occurring Glycosides* (pp. 225-274). Chichester: John Wiley and Sons Ltd.

Crouzet, J., and Kanasawud, P. (1992). Formation of volatile compounds by thermal degradation of carotenoids. In *Methods in Enzymology* (Vol. 213, pp. 54-62).

Crouzet, J., Kanasawud, P., and Sakho, M. (2002). Thermal generation of carotenoid-derived compounds. In P. Winterhalter & R. L. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 115-130). Washington DC: American Chemical Society.

Culleré, L., Escudero, A., Cacho, J., and Ferreira, V. (2005). Gas chromatography-olfactometry and chemical quantitative study of the aroma of six premium quality Spanish aged red wines. *Journal of Agricultural and Food Chemistry, **52**,* 1653-1660.

Czeczuga, B., Moburg, R., and Alstrup, V. (1992). Studies on carotenoids in lichens. XXXII. Carotenoids in the thalli of lichens from Kenya. *Acta Societas Botanicorum Poloniae, **61**,* 231-239.

D'alvise, N., Leseur-Lambert, C., Fertin, B., and Dhulster, P. (2000). Removal of polyphenols and recovery of proteins from alfalfa white protein concentrate by ultrafiltration and adsorbent resin separations. *Separation Science and Technology, **35***, 2453-2472.

Davies, N. W., and Menary, R. C. (1983). Volatile constituents of *Boronia megastigma* flowers. *Perfumer and Flavorist, **8***, 3-8.

De Tommasi, N., Piacente, S., De Simone, F., and Pizza, C. (1996). Constituents of *Cydonia vulgaris*: Isolation and structure elucidation of four new flavonol glycosides and nine new β-ionol-derived glycosides. *Journal of Agricultural and Food Chemistry, **44***, 1676-1681.

Dembitsky, V. M. (2004). Chemistry and biodiversity of the biologically active natural glycosides. *Chemistry and Biodiversity, **1***, 673-781.

Demyttenaere, J. C. R. (2001). Biotransformation of terpenoids by microorganisms. *Studies in Natural Products Chemistry, **25***, 125-178.

Dewick, P. M. (2002). The biosynthesis of C_5-C_{25} terpenoid compounds. *Natural product Reports, **19***, 181-222.

D'Incecco, N., Bartowsky, E., Kassara, S., Lante, A., Spettoli, P., and Henschke, P. (2004). Release of glycosidically bound flavour compounds of chardonnay by *Oenococcus oeni* during malolactic fermentation. *Food Microbiology, **21***, 257-265.

Doner, L. W., Becard, G., and Irwin, P. L. (1993). Binding of flavonoids by polyvinylpolypyrrolidone. *Journal of Agricultural and Food Chemistry, **41***, 753-757.

Dudareva, N., Pichersky, E., and Gershenzon, J. (2004). Biochemistry of plant volatiles. *Plant Physiology, **135***, 1893-1902.

Duque, C., Osorio, C., and Morales, A. (2002). C13-Norisoprenoids in the aroma of Colombian tropical fruits. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 194-205). Washington DC: American Chemical Society.

Edge, R., McGarvey, D. J., and Truscott, T. G. (1997). The carotenoids as anti-oxidants - a review. *Journal of Photochemistry and Photobiology B: Biology, **41***, 189-200.

Eisenreich, W., Bacher, A., Arigoni, D., and Rohdich, F. (2004). Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cellular and Molecular Life Sciences, **61***, 1401-1426.

Engel, K.-H., and Tressl, R. (1983). Formation of aroma components from nonvolatile precursors in passion fruit. *Journal of Agricultural and Food Chemistry, **31***, 998-1002.

Enzell, C. (1985). Biodegradation of carotenoids - an important route to aroma compounds. *Pure and Applied Chemistry, **57***, 693-700.

Enzell, C. R., and Back, S. (1995). Mass Spectrometry. In G. Britton, S. Liaaen-Jensen & H. Pfander (Eds.), *Carotenoids Volume 1B: Spectroscopy* (Vol. 1B, pp. 261-320). Basel: Birkhauser Verlag.

Etievant, P. X., Issanchou, S. N., and Bayonove, C. L. (1983). The flavour of Muscat wine: The sensory contribution of some volatile compounds. *Journal of the Science of Food and Agriculture, **34***, 497-504.

Eugster, C. H., and Marki-Fischer, E. (1991). The chemistry of rose pigments. *Angewandte Chemie-International Edition in English, **30***, 654-672.

Fester, T., Schmidt, D., Lohse, S., Walter, M., Giuliano, G., Bramley, P. M., Fraser, P. D., Hause, B., and Strack, D. (2002). Stimulation of carotenoid metabolism in arbuscular mycorrhizal roots. *Planta, **216***, 148-154.

Fitz, W., Kerler, J., and Weenen, H. (1999). Lipid derived flavours. In K. A. D. Swift (Ed.), *Current Topics in Flavours and Fragrances. Towards a New Millennium of Discovery* (pp. 171-214). Dordrecht: Kluwer Academic Publishers.

Flament, I. (1991). Coffee, Cocoa and Tea. In H. Maarse (Ed.), *Volatile Compounds in Foods and Beverages.* (pp. 617-669). New York: Marcel Dekker, Inc.

Fleischmann, P., Studer, K., and Winterhalter, P. (2002). Partial purification and kinetic characterisation of a carotenoid cleavage enzyme from quince fruit (*Cydonia oblonga*). *Journal of Agricultural and Food Chemistry, **50***, 1677-1680.

Fleischmann, P., Watanabe, N., and Winterhalter, P. (2003). Enzymatic carotenoid cleavage in star fruit (*Averrhoa carambola*). *Phytochemistry, **63***, 131-137.

Francis, M. J. O., and Allcock, C. (1969). Geraniol β-D-glucoside; Occurrence and synthesis in rose flowers. *Phytochemistry, **8***, 1339-1347.

Friedrich, J. E., and Acree, T. E. (2002). Analysing carotenoid-derived aroma compounds using gas chromatography - olfactometry. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 67-75). Washington DC: American Chemical Society.

Fujimori, T., Kasuga, R., Matsushita, H., Kaneko, H., and Noguchi, M. (1976). Neutral aroma constituents in burley tobacco. *Agricultural and Biological Chemistry, **40***(2), 303-315.

Fundira, M., Blom, M., Pretorius, I. S., and Van Rensberg, P. (2002). Comparison of commercial enzymes for the processing of marula pulp, wine and spirits. *Journal of Food Science, **67***(6), 2346-2351.

Gansser, D., Latza, S., and Berger, R. G. (1997). Methyl jasmonates in developing strawberry fruit (*Fragaria ananassa* Duch. Cv. Kent). *Journal of Agricultural and Food Chemistry, **45***, 2477-2480.

Georgiev, E. V., and Ballinova-Tsvetkova, A. (1977). *A comparative study of some solvents for the extraction of lavender racemes.* Paper presented at the VIIth International Congress of Essential Oils., Kyoto, Japan.

Ghisalberti, E. L. (1998). Phytochemistry of the Australian Rutaceae: *Boronia*, *Eriostemon* and *Phebalium* species. *Phytochemistry, **47***(2), 163-176.

Gholami, M., Coombe, B. G., Robinson, S. P., and Williams, P. J. (1996). Amounts of glycosides in grapevine organs during berry development. *Australian Journal of Grape and Wine Research, **2***, 59-63.

Gibson, A. M., and Hocking, A. D. (1997) Advances in the predictive modelling of fungal growth in food. *Trends in Food Science and Technology*, ***8***, 353-358.

Giuliano, G., Al-Babili, S., and von Lintig, J. (2003). Carotenoid oxygenases: cleave it or leave it. *Trends in Plant Science, **8***(4), 145-149.

Godoy, H. T., and Rodríguez-Amaya, D. B. (1994). Occurrence of *cis*-isomers of provitamin A in Brazilian fruits. *Journal of Agricultural and Food Chemistry, **42***, 1306-1313.

Goodwin, T. W. (1993). Biosynthesis of carotenoids: An overview. In L. Packer (Ed.), *Methods in Enzymology* (Vol. 214 Carotenoids Part B Metabolism, Genetics, and Biosynthesis, pp. 330-340). San Diego: Academic Press, Inc.

Grosch, W., and Schieberle, P. (1991). Bread. In H. Maarse (Ed.), *Volatile Compounds in Foods and Beverages* (pp. 41-77). New York: Marcel Dekker, Inc.

Grosch, W., Weber, F., and Fischer, K. H. (1977). Bleaching of carotenoid by the enzyme lipoxygenase. *Annales de Technologie Agricole, **26***, 133-137.

Gross, J., Gabai, M., and Lifshitz, A. (1972). A comparative study of the carotenoid pigments in juice of shamouti, valencia, and washington oranges. *Phytochemistry, **11***, 303-308.

Groyne, J., Lognay, G., and Marlier, M. (1999). Accumulation of glycosidically bound compounds in *Fragaria* x *ananassa* cv, Elsanta fruits at various developmental stages. *Biotechnology, Agronomy, Society and Environment.*

Grün, I., U., Adhikari, K., Li, C., Li, Y., Lin, B., Zhang, J., and Fernando, L. N. (2001). Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of tofu. *Journal of Agricultural and Food Chemistry, **49***, 2839-2843.

Guenther, E. (1949). Oil of *Boronia megastigma*. In *The Essential Oils* (Vol. 3, pp. 364-367). New York: Robert E. Krieger Publishing Co. Inc.

Günata, Y. Z., Bayonove, C. L., Baumes, R. L., and Cordonnier, R. E. (1985). The aroma of grapes. 1. Extraction and determination of free and glycosidically bound fractions of some grape aroma compounds. *Journal of Chromatography, **331***, 83-90.

Günata, Z., Wirth, J. L., Guo, W., and Baumes, R. L. (2002). C-13-Norisoprenoid aglycon composition of leaves and grape berries from Muscat of Alexandria and Shiraz cultivars. In P. Winterhalter & R. L. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 255-261). Washington DC: American Chemical Society.

Guo, W., Sakata, K., Watanabe, N., Nakajima, R., Yagi, A., Ina, K., and Luo, S. (1993). Geranyl 6-O-β-D-xylopyranosyl-β-D-glucopyranoside isolated as an aroma precursor from tea leaves for oolong tea. *Phytochemistry, **33***(6), 1373-1375.

Hartman, D. A., Pontones, M. E., Kloss, V. F., Curley, R. W., and Robertson, L. W. (1988). Models of retinoid metabolism: Microbial biotransformation of α-ionone and β-ionone. *Journal of Natural Products, **51***(5), 947-953.

Hawken, P., Lovins, A. B., and Lovins, L. H. (1999). *Natural Capitalism. The Next Industrial Revolution.* London: Earthscan Publications Ltd.

Henry, L. K., Catignani, G. L., and Schwartz, S. J. (1998). Oxidative degradation kinetics of lycopene, lutein, and 9-cis and all-trans β-carotene. *Journal of the American Oil Chemist's Society, **75***, 823-829.

Herderich, M. (2002). HPLC-MS analysis of carotenoid-derived aroma precursors. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Precursors* (Vol. 36-55). Washington DC: American Chemical Society.

Hewitt, E. J., Mackay, D. A. M., Konigsbacher, K., and Hasselstrom, T. (1956). The role of enzymes in food flavors. *Food Technology, **10***, 487-489.

Hisamoto, M., Kikuzaki, H., and Nakatani, N. (2004). Constituents of the leaves of *Peucedanum japonicum* Thunb. and their biological activity. *Journal of Agricultural and Food Chemistry, **52***, 445-450.

Holstein, S. A., and Hohl, R. J. (2004). Isoprenoids: Remarkable Diversity of form and function. *Lipids, **39***, 293-309.

Huang, H. T. (1956). The kinetics of the decolourisation of anthocyanins by fungal "anthocyanase". *Journal of the American Chemical Society, **78***, 2390-2393.

Humpf, H.-U., and Schreier, P. (1992). 3-Hydroxy-5,6-epoxy-β-ionol β-D-glucopyranoside and 3-hydroxy-7,8-dihydro-β-ionol β-D-glucopyranoside: New C-13 norisoprenoid glucoconjugates from Sloe Tree (*Prunus spinosa* L.) leaves. *Journal of Agricultural and Food Chemistry, **40***, 1898-1901.

Isoe, S., Hyeon, S. B., and Sakan, T. (1969). Photo-oxygenation of carotenoids. I. The formation of dihydroactinidiolide and β-ionone from β-carotene. *Tetrahedron Letters, 4*, 279-281.

Jarén-Galán, M., Carmona-Ramón, C., and Mínguez-Mosquera, M. I. (1999). Interaction between chloroplast pigments and lipoxygenase enzymatic extract of olives. *Journal of Agricultural and Food Chemistry, 47*, 2671-2677.

Juttner, F. (1988). Carotene oxygenase in *Microcystis.* In S. P. Colowick & N. O. Kaplan (Eds.), *Methods in Enzymology* (Vol. 167, pp. 336-341). New York: Academic Press.

Juttner, F., and Hoflacher, B. (1985). Evidence of β-carotene 7,8(7',8')oxygenase (β-cyclocitral, crocetindial generating) in *Microcystis. Archives of Microbiology, 141*, 337-343.

Kaiser, R. (2002). Carotenoid-Derived Aroma Compounds in Flower Scents. In P. Winterhalter & R. L. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 162-182). Washington DC: American Chemical Society.

Kanakis, C. D., Daferera, D. J., Tarantilis, P. A., and Polissiou, M. G. (2004). Qualitative determination of volatile compounds and quantitative evaluation of safranal and 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek saffron. *Journal of Agricultural and Food Chemistry, 52*, 4515-4521.

Khachik, F., Beecher, G. R., and Lushby, W. R. (1989). Separation, identification, and quantification of the major carotenoids in extracts of apricots, peaches, cantaloupe, and pink grapefruit by liquid chromatography. *Journal of Agricultural and Food Chemistry, 37*, 1465-1473.

Kilic, A., Kollmannsberger, H., and Nitz, S. (2005). Glycosidically bound volatiles and flavor precursors in *Laurus nobilis* L. *Journal of Agricultural and Food Chemistry, 53*, 2231-2235.

Knapp, H., Straubinger, M., Stingl, C., and Winterhalter, P. (2002). Analysis of norisoprenoid aroma precursors. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Precursors.* (pp. 20-35). Washington DC: American Chemical Society.

Krammer, G., Winterhalter, P., Schwab, M., and Schreier, P. (1991). Glycosidically bound aroma compounds in the fruits of *Prunus* species: Apricot (*P. ermeniaca*, L.), Peach (*P. persica*, L.), Yellow Plum (*P. domestica*, L. ssp. Syriaca). *Journal of Agricultural and Food Chemistry, 39*, 778-781.

Kreck, M., Puschel, S., Wust, M., and Mosandl, A. (2003). Biogenetic studies in *Syringa vulgaris* L.: Synthesis and bioconversion of deuterium labeled precursors into lilac aldehydes and lilac alcohols. *Journal of Agricultural and Food Chemistry, 51*, 463-469.

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K. (1991). Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). *Agricultural and Biological Chemistry, **55***(9), 2227-2233.

Kull, D., and Pfander, H. (1995). Appendix: List of New Carotenoids. In G. Britton, S. Liaaen-Jensen & H. Pfander (Eds.), *Carotenoids: Isolation and Analysis* (Vol. 1A, pp. 295-317). Basel: Birkhauser.

Kuzuyama, T. (2002). Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Bioscience, Biotechnology and Biochemistry, **66***, 1619-1627.

Lalel, H. J. D., Singh, Z., and Tan, S. C. (2003). Glycosidically-bound aroma volatile compounds in the skin and pulp of 'Kensington Pride' mango fruit at different stages of maturity. *Postharvest Biology and Technology, **29***, 205-218.

Larroche, C., Creuly, C., and J.-B., G. (1995). Fed-batch biotransformation of β-ionone by *Aspergillus niger*. *Applied Microbiology and Biotechnology, **43***, 222-227.

Latrasse, A. (1991). Fruits III. In H. Maarse (Ed.), *Volatile Compounds in Foods and Beverages*. New York: Marcel Dekker, Inc.

Lermusieau, G., Bulens, M., and Collin, S. (2001). Use of GC-Olfactometry to identify the hop aromatic compounds in beer. *Journal of Agricultural and Food Chemistry, **49***, 3867-3874.

Lewinsohn, E., Sitrit, Y., Bar, E., Azulay, Y., Ibdah, M., Meir, A., Yosef, E., Zamir, D., and Tadmor, Y. (2005). Not just colors - carotenoid degradation as a link between pigmentation and aroma in tomato and watermelon fruit. *Trends in Food Science and Technology, **16***, 407-415.

Liaaen-Jensen, S. (1995). Combined approach: Identification and structure elucidation of carotenoids. In G. Britton, S. Liaaen-Jensen & H. Pfander (Eds.), *Carotenoids Volume 1B: Spectroscopy* (Vol. 1B, pp. 343-354). Basel: Birkhauser Verlag.

López, R., Ferreira, V., Hernández, P., and Cacho, J. F. (1999). Identification of impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties: a comparative study. *Journal of the Science of Food and Agriculture, **79***, 1461-1467.

Lutz, A., and Winterhalter, P. (1992). Isolation of additional carotenoid metabolites from quince fruit (*Cydonia oblonga* Mill.). *Journal of Agricultural and Food Chemistry, **40***, 1116-1120.

Lutz-Röder, A., Schneider, M., and Winterhalter, P. (2002). Isolation of two new ionone glucosides from quince (*Cydonia oblonga* Mill.) leaves. *Natural Product Letters, **16***(2), 119-122.

Ma, S.-J., Watanabe, N., Yagi, A., and Sakata, K. (2001). The (3R,9R)-3-hydroxy-7,8-dihydro-β-ionol disaccharide glycoside is an aroma precursor in tea leaves. *Phytochemistry, **56***, 819-825.

MacTavish, H. (1995). *Factors affecting yield and composition of floral extract from Boronia megastigma Nees.* PhD thesis, University of Tasmania, Hobart.

MacTavish, H., and Menary, R. C. (1997). The effect of flower maturity and harvest timing on floral extract from *Boronia megastigma* (Nees). *Annals of Botany, **80***, 299-303.

MacTavish, H., and Menary, R. C. (1998a). Biosynthesis of volatiles in brown boronia flowers after harvest: Effect of harvest time and incubation conditions. *Annals of Botany, **81***, 83-89.

MacTavish, H., and Menary, R. C. (1998b). Optimizing solvent extraction of *Boronia megastigma* (Nees) flowers. *Journal of Essential Oil Research, **10***, 31-37.

MacTavish, H., and Menary, R. C. (1999a). Floral extract from four clones of *Boronia megastigma* (Nees) throughout flowering. *Journal of Horticultural Science & Biotechnology, **74***(1), 47-52.

MacTavish, H., and Menary, R. C. (1999b). Production of volatiles in brown boronia flowers after harvest. I: Effect of clonal type and incubation temperature. *Journal of Horticultural Science & Biotechnology, **74***(4), 436-439.

MacTavish, H., and Menary, R. C. (1999c). Production of volatiles in brown boronia flowers after harvest. II: Effect of oxygen consumption. *Journal of Horticultural Science & Biotechnology, **74***(4), 440-442.

MacTavish, H., and Menary, R. C. (2000). Production of volatiles in brown boronia flowers after harvest: Pilot-scale research. *Journal of Horticultural Science & Biotechnology, **75***(4), 455-458.

MacTavish, H., Menary, R. C., and Davies, N. W. (2002). Bound volatiles in brown boronia flowers (*Boronia megastigma*). In P. Winterhalter & R. L. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (Vol. 802, pp. 183-193). Washington DC: American Chemical Society.

Mahattanatwee, K., Rouseff, R., Valim, M. F., and Naim, M. (2005). Identification and aroma impact of norisoprenoids in orange juice. *Journal of Agricultural and Food Chemistry, **53***, 393-397.

Maicas, S., and Mateo, J. J. (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. *Applied Microbiology and Biotechnology, **67***, 322-335.

Martino, A., Pifferi, P. G., and Spagna, G. (1994). The separation of pectinlyase from β-glucosidase in a commercial preparation. *Journal of Chemical Technology and Biotechnology, **61***, 255-260.

Mateo, J. J., and Jiménez, M. (2000). Monoterpenes in grape juice and wines. *Journal of Chromatography A, **881***, 557-567.

Matile, P., Hortensteiner, S., and Thomas, H. (1999). Chlorophyll degradation. *Annual Review of Plant Physiology, **50***, 67-95.

Mayorga, H., Knapp, H., Winterhalter, P., and Duque, C. (2001). Glycosidically bound flavor compounds of Cape Gooseberry (*Physalis peruviana* L.). *Journal of Agricultural and Food Chemistry, **49***, 1904-1908.

Mercadante, S. Z. (1999a). New carotenoids: Recent progress. *Pure and Applied Chemistry, **71***(12), 2263-2272.

Mercadante, S. Z. (1999b). Chromatographic separation of carotenoids. *Archives of Latinoamerican Nutrition, **49***, 52S-57S.

Miranda, E. J. F., Nogueira, R. I., Pontes, S. M., and Rezende, C. M. (2001). Odour-active compounds of banana passa identified by aroma extract dilution analysis. *Flavour and Fragrance Journal, **16***, 281-285.

Misra, G., Pavlostathis, S. G., Perdue, E. M., and Araujo, R. (1996). Aerobic biodegradation of selected monoterpenes. *Applied Microbiology and Biotechnology, **45***, 831-838.

Mookherjee, B. D., Trenkle, R. W., Wilson, R. A., Zampino, M. J., Everett, E. S., and Patel, S. M. (1995, 15 - 19 October). *Creation of fragrances using synthetic molecules in the light of natural products.* Paper presented at the Thirteenth international congress of flavours, fragrances and essential oils., Instanbul.

Moon, J.-H., Watanabe, N., Sakata, K., Inagaki, J., Yagi, A., Ina, K., and Luo, S. (1994). Linalyl β-D-glucopyranoside and its 6'-O-malonate as aroma precursors from *Jasminum sambac*. *Phytochemistry, **36***(6), 1435-1437.

Morales, A. L., and Duque, C. (2002). Free and glycosidically bound volatiles in the mamee apple (*Mammea americana*) fruit. *European Food Research and Technology, **215***, 221-226.

Moshanus, M. G., and Shaw, P. E. (1997). A research note. Flavor and chemical comparison of pasteurized and fresh valencia orange juices. *Journal of Food Quality, **20***, 31-40.

Müller, H. (1997). Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection. *Zeitschrift fur Lebensmittel Untersuchung und Forschung A - Food Research and Technology, **204***, 88-94.

Murai, Y., Kashimura, S., Tamezawa, S., Hashimoto, T., Takaoka, S., Asakawa, Y., Kiguchi, K., Murai, F., and Tagawa, M. (2001). Absolute configuration of (6S, 9S)-roseoside from *Polygonum hydropiper*. *Planta Medica, **67***, 480-481.

Negre, F., Kish, C. M., Boatright, J., Underwood, B., Shibuya, K., Wagner, C., Clark, D. G., and Dudareva, N. (2003). Regulation of methylbenzoate emission after pollination in snapdragon and petunia flowers. *The Plant Cell, **15***, 2992-3006.

Nishikitani, M., Kubota, K., Kobayashi, A., and Sugawara, F. (1996). Geranyl 6'-O-α-L-arabinopyranosyl-β-D-glucopyranoside isolated as an aroma precursor from leaves of a green tea cultivar. *Bioscience Biotechnology and Biochemistry, **60***(5), 929-931.

Nordstrom, C. G. (1967). Separation of polyphenolic glycosides by gel chromatography. *Acta Chemica Scandinavica, **21***, 2885-2912.

Odoux, E., Chauwin, A., and Brillouet, J.-M. (2003). Purification and characterisation of vanilla bean (*Vanilla planifolia* Andrews) β-D-glucosidase. *Journal of Agricultural and Food Chemistry, **51***, 3168-3173.

Oliver, J., and Palou, A. (2000). Chromatographic determination of carotenoids in foods. *Journal of Chromatography A, **881***, 543-555.

Osorio, C., Duque, C., and Batista-Viera, F. (2003). Studies on aroma generation in lulo (*Solanum quitoense*): enzymatic hydrolysis of glycosides from leaves. *Food Chemistry, **81***, 333-340.

Osorio, C., Duque, C., and Fujimoto, Y. (1999). C-13 Norisoprenoid glucoconjugates from Lulo (Solanum quitoense L.) leaves. *Journal of Agricultural and Food Chemistry, **47***, 1641-1645.

Pabst, A., Barron, D., Etievant, P. X., and Schreier, P. (1991). Studies on the enzymatic hydrolysis of bound aroma constituents from raspberry fruit pulp. *Journal of Agricultural and Food Chemistry, **39***, 173-175.

Pabst, A., Barron, D., Semon, E., and Schreier, P. (1992). A 4-hydroxy-β-ionone disaccharide glycoside from raspberry fruits. *Phytochemistry, **31***(9), 3105-3107.

Parada, F., Duque, C., and Fujimoto, Y. (2000). Free and bound volatile composition and characterization of some glucoconjugates as aroma precursors in Melon de Olor fruit pulp (*Sicana odorifera*). *Journal of Agricultural and Food Chemistry, **48***, 6200-6204.

Plummer, J. A., Wann, J. M., and Spadek, Z. E. (1999). Intraspecific variation in oil components of *Boronia megastigma* Nees. (Rutaceae) flowers. *Annals of Botany, **83***, 253-262.

Pogolrel'skaya, A. N., Kholodova, V. P., and Reznikova, S. A. (1979). Physiological aspects of essential oil accumulation in petals of the flowers of essential-oil rose. *Fiziologiya Rastenii, **27***, 356-362.

Raguso, R. A., and Pichersky, E. (1999). A day in the life of a linalool molecule: Chemical communication in a plant-pollinator system. Part 1: Linalool biosynthesis in flowering plants. *Plant Species Biology, **14***, 95-120.

Rega, B., Fournier, N., and Guichard, E. (2003). Solid phase microextraction (SPME) of orange juice flavor: Odor representativeness by direct gas chromatography olfactometry (D-GC-O). *Journal of Agricultural and Food Chemistry, **51***, 7092-7099.

Roberts, D. D., Mordehai, A. P., and Acree, T. E. (1994). Detection and partial characterisation of eight β-damascenone precursors in apples (*Malus domestica Borkh.* Cv. Empire). *Journal of Agricultural and Food Chemistry, **42***, 345-349.

Rodríguez-Bustamante, E. R., Maldonado-Robledo, G., Ortiz, M. A., Díaz-Ávalos, C., and Sanchez, S. (2005). Bioconversion of lutein using a microbial mixture-maximizing the production of tobacco aroma compounds by manipulation of culture medium. *Applied Microbiology and Biotechnology, **68***, 174-182.

Roscher, R., Herderich, M., Steppen, J.-P., Schreier, P., and Schwab, W. (1996). 2,5-Dimethyl-4-hydroxy-3(2H)-furanone 6'-O-malonyl-β-D-glucopyranoside in strawberry fruits. *Phytochemistry, **43***(1), 155-159.

Roscher, R., Schwab, W., and Schreier, P. (1997). Stability of naturally occurring 2,5-dimethyl-4-hydroxy-3(2H)-furanone derivatives. *Zeitschrift fuer Lebensmittel Untersuchung und Forschung, **204***, 438-441.

Rouseff, R. L., Martin, S. F., and Youtsey, C. O. (1987). Quantitative survey of narirutin, naringen, hesperidin, and neohesperidin in *citrus*. *Journal of Agricultural and Food Chemistry, **35***, 1027-1030.

Sánchez-Contreras, A., Jiménez, M., and Sanchez, S. (2000). Bioconversion of lutein to products with aroma. *Applied Microbiology and Biotechnology, **54***, 528-534.

Sanderson, G. W., and Gonzalez, J. G. (1971). Biochemistry of teas formation: The role of carotenes in black teas aroma formation. *Journal of Food Science, **36***, 231-236.

Sarry, J.-E., and Günata, Z. (2004). Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors. *Food Chemistry, **87***, 509-521.

Scharpf, J., L. G., Seitz, E. W., Morris, J. A., and Farbood, M. I. (1986). Generation of flavor and odor compounds through fermentation processes. In T. H. Parliment & R. Croteau (Eds.), *Biogeneration of Aromas* (pp. 323-346). Washington DC: American Chemical Society.

Schneider, R., Razungles, A., Augier, C., and Baumes, R. (2001). Monoterpenic and norisoprenoidic glycoconjugates of *Vitis vinifera* L. cv. Melon B. as precursors of odorants in Muscadet wines. *Journal of Chromatography A, **936***, 145-157.

Schreier, P. (1997). Enzymes and Flavour Biotechnology. In T. Scheper (Ed.), *Advances in Biochemical Engineering/Biotechnology* (Vol. 55, pp. 52-72). Heidelberg: Springer-Verlag.

Schulz, G., and Stahl-Biskup, E. (1991). Essential oils and glycosidic bound volatiles from leaves, stems, flowers and roots of *Hyssopus officinalis* L. (Lamiaceae). *Flavour and Fragrance Journal, **6***, 69-73.

Schwab, W., and Schreier, P. (1988). Aryl β-D-glucosides from *Carica papaya* fruit. *Phytochemistry, **27***, 1813-1816.

Schwartz, S. H., Qin, X., and Zeevaart, J. A. D. (2001). Characterisation of a novel carotenoid cleavage dioxygenase from plants. *The Journal of Biological Chemistry, **276***(27), 25208-25211.

Schwartz, S. H., Tan, B. C., Gage, D. A., Zeevaart, J. A. D., and McCarty, D. R. (1997). Specific oxidative cleavage of carotenoids by VP14 of maize. *Science, **276***, 1872-1874.

Scordino, M., Di Mauro, A., Passerini, A., and Maccarone, E. (2005). Selective recovery of anthocyanins and hydroxycinnamates from a byproduct of citrus processing. *Journal of Agricultural and Food Chemistry, **53***, 651-658.

Sefton, M. A., and Williams, P. J. (1991). Generation of oxidation artifacts during the hydrolysis of norisoprenoid glycosides by fungal enzyme preparations. *Journal of Agricultural and Food Chemistry, **39***, 1994-1997.

Seto, H., Nomura, E., Fujioka, S., Koshino, H., Suenaga, T., and Yoshida, S. (1999). Easy preparation of methyl 7-epi-jasmonate and four stereoisomers of methyl cucurbate, and assessment of the stereogenic effect of jasmonate on phytohormonal activities. *Bioscience, Biotechnology and Biochemistry, **63***(2), 361-367.

Sharma, R. V., Mathur, N., Dmitrovskii, A. A., Das, R. C., and Ganguly, J. (1977). Studies on the metabolism of β-carotene and apo-β-carotenoids in rats and chickens. *Biochimica et Biophysica Acta, **486***, 183-194.

Simkin, A. J., Schwartz, S. H., Auldridge, M., Taylor, M. G., and Klee, H. J. (2004). The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles -ionone, pseudoionone, and geranylacetone. *The Plant Journal, **40***, 882-892.

Simpson, K. L., and Chichester, C. O. (1981). Metabolism and nutritional significance of carotenoids. *Annual Review of Nutrition, **1***, 351-374.

Singh, H., John, J., and Cama, H. R. (1973). Separation of β-apocarotenals and related compounds by reversed-phase paper and thin-layer chromatography. *Journal of Chromatography, **75***, 146-150.

Skouroumounis, G. K., Günata, Y. Z., and Baumes, R. L. (2000). Isolation of two megastigmane-3,9-diol glucosides from Shiraz leaves. *Journal of Essential Oil Research, **12***, 653-660.

Skouroumounis, G. K., and Sefton, M. A. (2000). Acid-catalyzed hydrolysis of alcohols and their β-D-glucopyranosides. *Journal of Agricultural and Food Chemistry, **48***, 2033-2039.

Spagna, G., Barbagallo, R. N., Martino, A., and Pifferi, P. G. (2000). A simple method of for purifying glycosidases: α-L-rhamnopyranosidase from *Aspergillus niger* to increase the aroma of Moscato wine. *Enzyme and Microbial Technology, **27***, 522-530.

Spagna, G., Romagnoli, D., Angela, M., Bianchi, G., and Pifferi, P. G. (1998). A simple method for purifying glycosidases: α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* to increase the aroma of wine. Part I. *Enzyme and Microbial Technology, **22***, 298-304.

Stahl-Biskup, E. (1987). Monoterpene glycosides, State-of-the-art. *Flavour and Fragrance Journal, **2***, 75-82.

Stahl-Biskup, E., Intert, F., Holthuijzen, J., Stengele, M., and Schulz, G. (1993). Glycosidically bound volatiles - A review 1986 - 1991. *Flavour and Fragrance Journal, **8***, 61-80.

Stevens, M. A. (1970). Relationship between polyene-carotene content and volatile compound composition of tomatoes. *Journal of the American Society for Horticultural Science, **95***, 461-464.

Stingl, C., Knapp, H., and Winterhalter, P. (2002). 3,4-Dihydroxy-7,8-dihydro-β-ionne 3-O-β-D-glucopyranoside and other glycosidic constituents from apple leaves. *Natural Product Letters, **16***(2), 87-93.

Strack, D., Fester, T., Hause, B., Schliemann, W., and Walter, M. H. (2003). Arbuscular Mycorrhiza: Biological, chemical and molecular aspects. *Journal of Chemical Ecology, **29***, 1955-1979.

Stratton, S. P., Schaefer, W. H., and Liebler, D. C. (1993). Isolation and identification of singlet oxygen oxidation products of β-carotene. *Chemical Research in Toxicology, **6***, 542-547.

Straub, O. (1987). *Key to carotenoids* (2 ed.). Basel: Birkhauser Verlag.

Straubinger, M., Knapp, H., Oka, N., Watanabe, N., and Winterhalter, P. (1997). Isolation of a glucosidic-damascenone precursor from rose petals. *Journal of Agricultural and Food Chemistry, **45***, 4053-4056.

Strauss, C. R., Wilson, B., Gooley, P. R., and Williams, P. J. (1986). Role of monoterpenes in grape and wine flavor. In T. H. Parliment & R. Croteau (Eds.), *Biogeneration of Aromas* (pp. 222-242). Washington DC: American Chemical Society.

Suárez, M., Duque, C., Wintoch, H., and Schreier, P. (1991). Glycosidically bound aroma compounds from the pulp and the peelings of Lulo Fruit (*Solanum vestissimum* D.). *Journal of Agricultural and Food Chemistry, **39***, 1643-1645.

Subarnas, A., Tadano, T., Oshima, Y., Kisara, K., and Ohizumi, Y. (1993). Pharmacological properties of beta-amyrin palmitate, a novel centrally acting compound, isolated from *Lobelia inflata* leaves. *Journal of Pharmacy and Pharmacology, **45***, 545-550.

Suzuki, M., Matsumoto, S., Fleischmann, H.-P., Shimada, H., Yamano, Y., Ito, M., and Watanabe, N. (2002). Identification of β-damascenone progenitors and their biogenesis in rose flowers (*Rosa damascena* Mill.). In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 89-101). Washington DC: American Chemical Society.

Tamborra, P., Martino, N., and Esti, M. (2004). Laboratory tests on glycosidase preparations in wine. *Analytica Chimica Acta, **513***, 299-303.

Tan, B.-C., Joseph, L. M., Deng, W.-T., Liu, L., Li, Q.-B., Cline, K., and McCarty, D. R. (2003). Molecular characterisation of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase gene family. *The Plant Journal, **35***, 44-56.

Tan, Y., and Siebert, K. J. (2004). Quantitative structure-activity relationship modelling of alcohol, ester, aldehyde, and ketone flavor thresholds in beer from molecular features. *Journal of Agricultural and Food Chemistry, **52***, 3057-3064.

van Breeman, R. B. (1997). Liquid chromatography/mass spectrometry of carotenoids. *Pure and Applied Chemistry, **69***(10), 2061-2066.

van Breeman, R. B., Huang, C.-R., Tan, Y., Sander, L. C., and Schilling, A. B. (1996). Liquid chromatography/mass spectrometry of carotenoids using atmospheric pressure chemical ionization. *Journal of Mass Spectrometry, **31***, 975-981.

Van den Dries, J. M. A., Svendsen, A., and Baerheim, A. (1989). A simple method for the detection of glycosidic bound monoterpenes and other volatile compounds occurring in fresh plant material. *Flavour and Fragrance Journal, **4***, 59-61.

van Vliet, T., van Schaik, F., Schreurs, W. H. P., and van den Berg, H. (1996). *In vitro* measurement of β-carotene cleavage activity: Methodological considerations and the effect of other carotenoids on β-carotene cleavage. *International Journal for Vitamin and Nutrition Research, **66***, 77-85.

Vernin, G., Vernin, C., Pieribattesti, J. C., and Roque, C. (1998). Analysis of the volatile compounds of *Psidium cattleianum* Sabine fruit from the Reunion Island. *Journal of Essential Oil Research, **10***, 353-362.

Verpoorte, R., Contin, A., and Memelink, J. (2002). Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews, **1***, 13-25.

Waché, Y., and Belin, J.-M. (2000). *Obtention de damascenone par voie biotechnologique, mythe ou realite?* Paper presented at the Rencontres AGORAL, Montpelier.

Waché, Y., Bosser-De Ratuld, A., and Belin, J.-M. (2002). Production of aroma compounds by enzymatic cooxidation of carotenoids. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 102-113). Washington DC: American Chemical Society.

Wahid, A., Bukhari, S., and Rasul, E. (2003). Inter-specific differences in cotton for nutrient partitioning from subtending leaves to reproductive parts at various developmental stages: consequences for fruit growth and yield. *Biologia Plantarum, **47***, 379-385.

Wahlberg, I., and Eklund, A.-M. (1998). Degraded carotenoids. In G. Britton, S. Liaaen-Jensen & H. Pfander (Eds.), *Carotenoids. Volume 3: Biosynthesis and Metabolism* (Vol. 3, pp. 195-216). Basel: Birkhauser Verlag.

Wang, D., Kurasawa, E., Yamaguchi, Y., Kubota, K., and Kobayashi, A. (2001). Analysis of glycosidically bound aroma precursors in tea leaves. 2. Changes in glycoside contents and glycosidase activities in tea leaves during black tea manufacturing process. *Journal of Agricultural and Food Chemistry, **49***, 1900-1903.

Wang, D., Yoshimura, T., Kubota, K., and Kobayashi, A. (2000). Analysis of glycosidically bound aroma precursors in tea leaves. 1. Qualitative and quantitative analyses of glycosides with aglycons as aroma compounds. *Journal of Agricultural and Food Chemistry, **48***, 5411-5418.

Wang, M., Shao, Y., Huang, T.-C., Wei, G.-J., and Ho, C.-T. (1998). Isolation and structural elucidation of aroma constituents bound as glycosides from sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry, **46***, 2509-2511.

Wang, Y., Finn, C., and Qian, M. C. (2005). Impact of growing environment on chickasaw blackberry (*Rubus* L.) aroma evaluated by gas chromatography olfactometry dilution analysis. *Journal of Agricultural and Food Chemistry, **53***, 3563-3571.

Watanabe, N. (1997). Enzymatic fragrance formation during flower opening. *Recent Research Developments in Agricultural and Biological Chemistry, **1***, 101-112.

Watanabe, N., Watanabe, S., Nakajima, R., Moon, J.-H., Shimokihara, K., Inagaki, J., Etoh, H., Asai, T., Sakata, K., and Ina, K. (1993). Formation of flower fragrance compounds from their precursors by enzymic action during flower opening. *Bioscience Biotechnology and Biochemistry, **57***(1), 1101-1106.

Wei, S., Marton, I., Shalitin, D., Lewinsohn, E., Bravdo, B.-A., and Shoseyov, O. (2004). Manipulating volatile emission in tobacco leaves by expressing *Aspergillus niger* β-glucosidase in different subcellular compartments. *Plant Biotechnology Journal, **2***, 341-350.

Weyerstahl, P., Marschall, H., Bork, W.-R., and Rilk, R. (1994). Megastigmanes and other constituents of the absolute of *Boronia megastigma* from Tasmania. *Liebigs Annalen der Chemie*, 1043-1047.

Weyerstahl, P., Marschall, H., Bork, W.-R., Rilk, R., Schneider, S., and Wahlburg, H.-C. (1995). Constituents of the absolute of *Boronia megastigma* Nees. from Tasmania. *Flavour and Fragrance Journal. **10***, 297-311.

Wightman, J. D., Price, S. F., Watson, B. T., and Wrolstad, R. E. (1997). Some effects of processing enzymes on anthocyanins and phenolics in Pinot Noir and Cabernet Sauvignon wines. *American Journal of Enology and Viticulture, **48***(1), 39-48.

Williams, P. J., Sefton, M. A., and Francis, I. L. (1992). Glycosidic precursors of varietal grape and wine flavour. In R. Teranishi, G. R. Takeoka & M. Guntert (Eds.), *Flavour Precursors; Thermal and Enzymatic Conversions* (Vol. 490, pp. 74-86). Washington DC: American Chemical Society.

Williams, P. J., Strauss, C. R., and Wilson, B. (1980). Hydroxylated linalool derivatives as precursors of volatile monoterpenes of Muscat grapes. *Journal of Agricultural and Food Chemistry, **28***, 766-771.

Williams, P. J., Strauss, C. R., Wilson, B., and Massy-Westropp, R. A. (1982). Studies on the hydrolysis of *Vitis vinifera* monoterpene precursor compounds and model monoterpene β-D-glucosides rationalizing the monoterpene composition of grapes. *Journal of Agricultural and Food Chemistry, **30***, 1219-1223.

Winkel, C., and Boesveld, M. (2000). Influence of a commercial pectinase preparation on tea flavour. In P. Schieberle & K.-H. Engel (Eds.), *Frontiers of Flavour Science* (pp. 366-369). Freising: Deutsche Forschungsanstalt fur Lebensmittelchemie, Garching.

Winterhalter, P. (1991). Fruits IV. In H. Maarse (Ed.), *Volatile Compounds in Foods and Beverages* (pp. 389-328). New York: Marcel Dekker, Inc.

Winterhalter, P., and Rouseff, R. (2002). Carotenoid-derived aroma compounds: An introduction. In P. Winterhalter & R. Rouseff (Eds.), *Carotenoid-Derived Aroma Compounds* (pp. 1-19). Washington DC: American Chemical Society.

Winterhalter, P., and Schreier, P. (1994). C-13 Norisoprenoid glycosides in plant tissues: an overview on their occurrence, composition and role as flavour precursors. *Flavour and Fragrance Journal, **9***, 281-287.

Winterhalter, P., and Schreier, P. (1995). The generation of norisoprenoid volatiles in starfruit (*Averrhoa carambola* L.): A review. *Food Reviews International, **11***, 237-254.

Winterhalter, P., and Skouroumounis, G. K. (1997). Glycoconjugated aroma compounds: Occurrence, role and biotechnological transformation. In R. G. Berger

(Ed.), *Biotechnology of Aroma Compounds* (Vol. 55, pp. 73-106). Berlin: Springer-Verlag.

Winterhalter, P., and Straubinger, M. (2000). Saffron a renewed interest in an ancient spice. *Food Reviews International, **16***, 39-59.

Wintoch, H., Morales, A., Duque, C., and Schreier, P. (1993). (R)-(-)-(E)-2,6-Dimethyl-3,7-octadiene-2,6-diol 6-O-β-Glucopyranoside: Natural precursor of hotrienol from Lulo fruit (*Solanum vestissimum* D.) peelings. *Journal of Agricultural and Food Chemistry, **41***, 1311-1314.

Wirth, J., Guo, W., Baumes, R. L., and Günata, Z. (2001). Volatile compounds released by enzymatic hydrolysis of glycoconjugates of leaves and grape berries from *Vitis vinifera* Muscat of Alexandria and Shiraz cultivars. *Journal of Agricultural and Food Chemistry, **48***, 2917-2923.

Wirth, J., Sauvage, F.-X., Baumes, R., and Günata, Z. (2003). Biogenesis of C-13 norisoprenoids in grape: biotransformation of C-13 norisoprenoids by a cell suspension culture of CV. Gamay. In J.-L. Le Quere & P. X. Etievant (Eds.), *Flavour Research at the Dawn of the Twenty-first Century.* (pp. 393-396). Paris: Intercept Ltd.

Withopf, B., Richling, E., Roscher, R., Schwab, W., and Schreier, P. (1997). Sensitive and selective screening for 6'-O-malonylated glucoconjugates in plants. *Journal of Agricultural and Food Chemistry, **45***, 907-911.

Wu, Z., Robinson, D. S., Hughes, R. K., Casey, R., Hardy, D., and West, S. L. (1999). Co-oxidation of β-carotene catalysed by soybean and recombinant pea lipoxygenases. *Journal of Agricultural and Food Sciences, **47***, 4899-4906.

Wyss, A. (2004). Carotene oxygenases: A new family of double bond cleavage enzymes. *Journal of Nutrition, **134 (1, Suppl.)***, 246S-250S.

Yadagiri, B., Raj, K., and Rao, G. S. R. S. (1984). Triterpenoids from *Balanophora abbreviata* and *Balanophora indica*. *Journal of Natural Products, **47***, 182-182.

Yokoyama, H., and White, M. J. (1966). Citrus carotenoids-VI. Carotenoid pigments in the flavedo of sinton citrangequot. *Phytochemistry, **5***, 1159-1173.

Zoecklein, B. W., Hackney, C. H., Duncan, S. E., and Marcy, J. E. (1999). Effect of fermentation, aging and thermal storage on total glycosides, phenol-free glycosides and volatile compounds of White Riesling (*Vitis vinifera* L.) wines. *Journal of Industrial Microbiology and Biotechnology, **22***, 100-107.

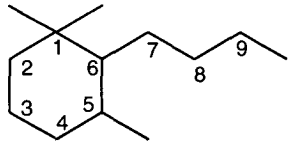
Zorn, H., Langhoff, S., Scheibner, M., Nimtz, M., and Berger, R. G. (2003). A peroxidase from *Lepista irinia* cleaves β,β-carotene to flavour compounds. *Biological Chemistry, **384***, 1049-1056.

Appendices

Appendix 1 – Notes with regard to the naming of C-13 norisoprenoids

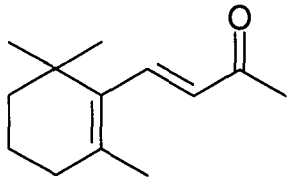
The naming of these compounds in the literature may be the source of some initial confusion. Particularly as the Weyerstahl publications have used trivial names. Whilst many of these names will be used in this document it is important to clarify the different naming systems. Winterhalter and Rouseff (2002) divide the C-13 norisoprenoid family into three groupings, namely ionones, damascones and megastigmanes and the ionol grouping has been added for completeness. **Figure A 1** details the primary members of each category and the numbering system used for carbon atoms. Confusion arises in the literature where members of the ionone family, as categorised by Winterhalter and Rouseff, are often named in the literature as megastigmanes. The numbering of the megastigmane carbons applies to all of the categories detailed in the figure. Details of alternative ionone and megastigmane names for three compounds commonly referred to in this thesis are included.

Figure A 1. Categorisation of naturally occurring C-13 norisoprenoids.



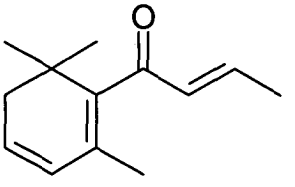
Megstigmane Carbon Skeleton

1. Ionones



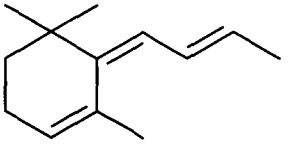
β-Ionone

2. Damascones



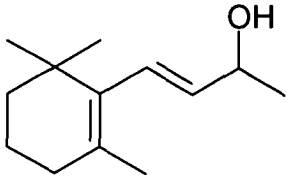
β-Damescenone

3. Megastigmanes



(E,E)-Megastigma-4,6,8-triene

4. Ionols



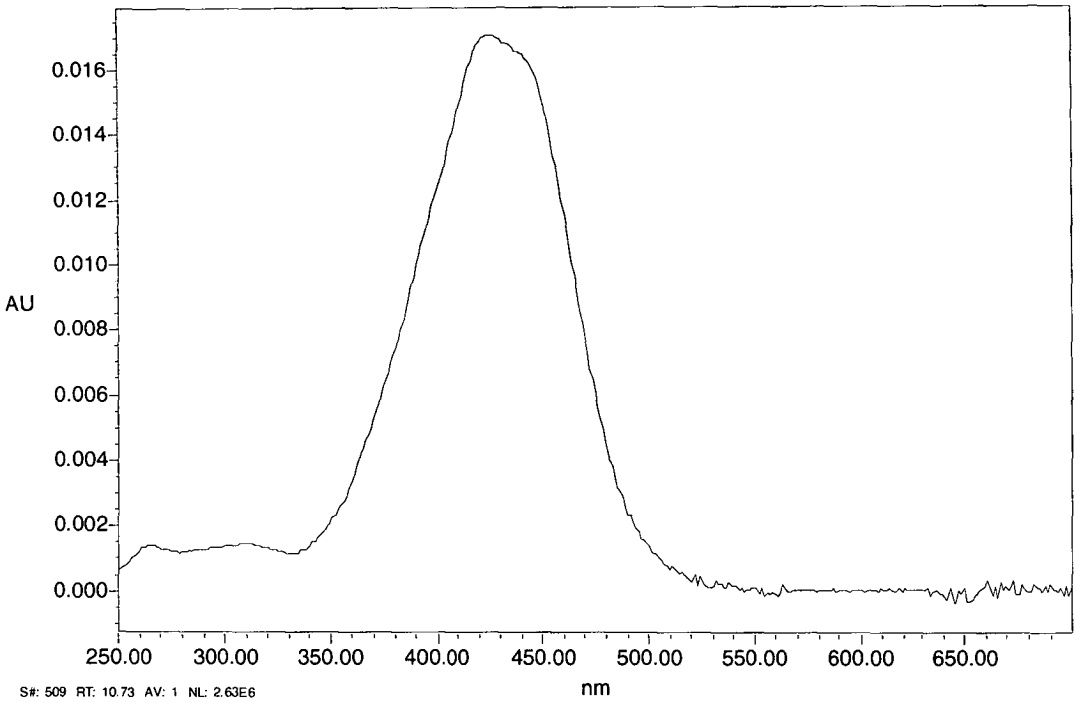
B-ionol

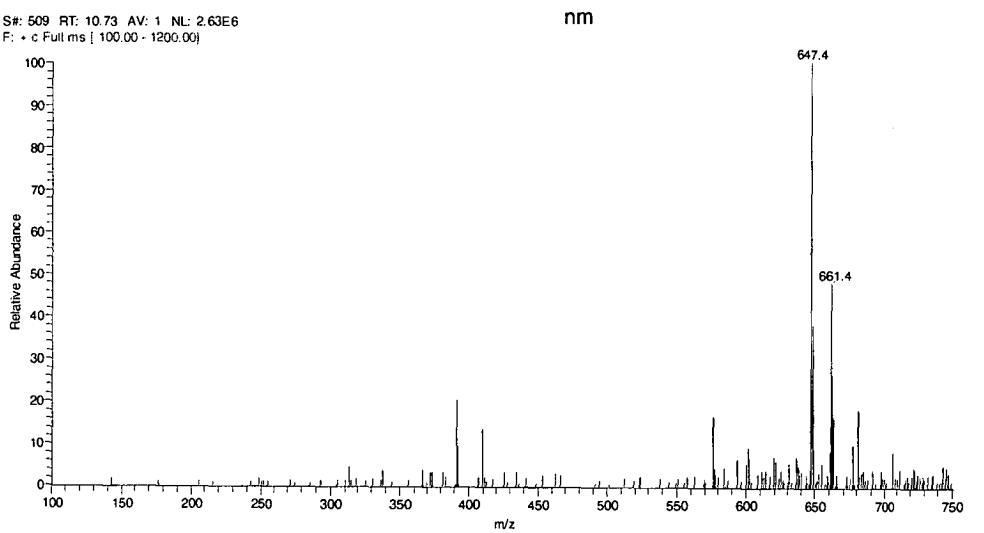
Ionone / Ionol	Megastigmane
3-hydroxy-5,6-dihydro-β-ionone	3-hydroxymegastigm-7-en-9-one
3-oxo-5,6-dihydro-β-ionone	megastigm-7-en-3,9-dione
3-oxo-5,6-dihydro-β-ionol	3-hydroxymegastigm-7-en-3-one

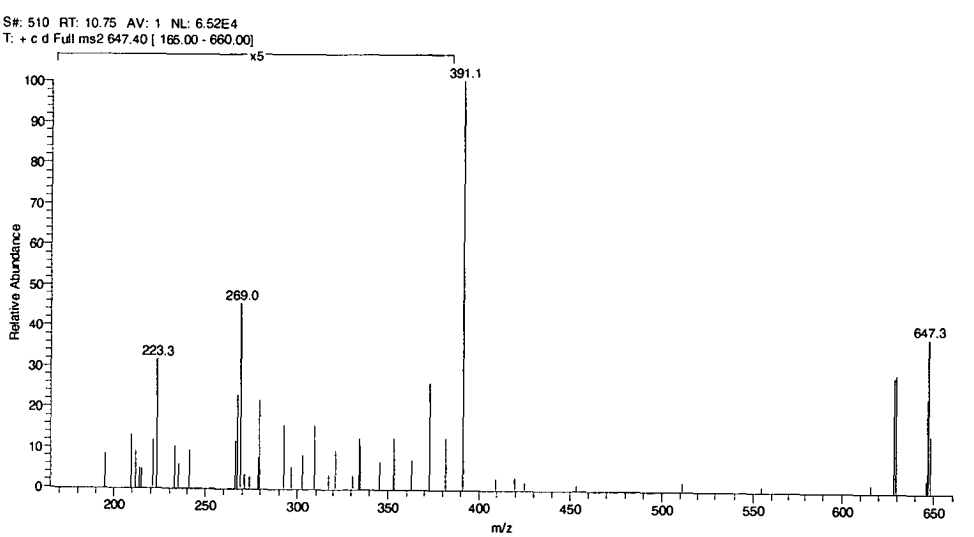
Appendix 2 – Data for the tentative palmitate ester of an apocarotenoid

Late apocarotenoid from boronia absolute (elutes near β-carotene) 10.757 mins on 'fast' program

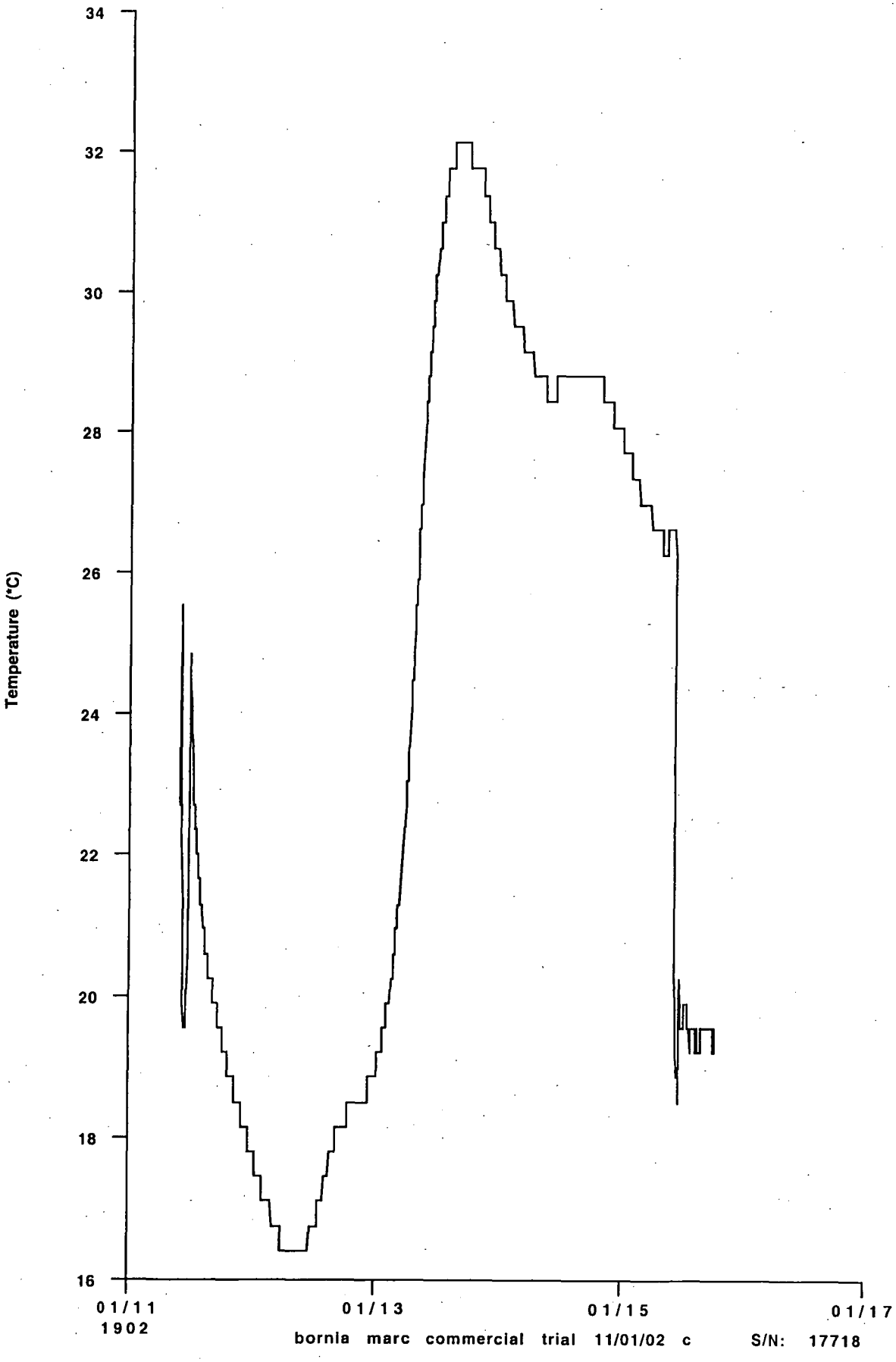
3-8-2000 fresh boronia absolute LCUVMS run / MS possibly $[M+H]^+$ 647,



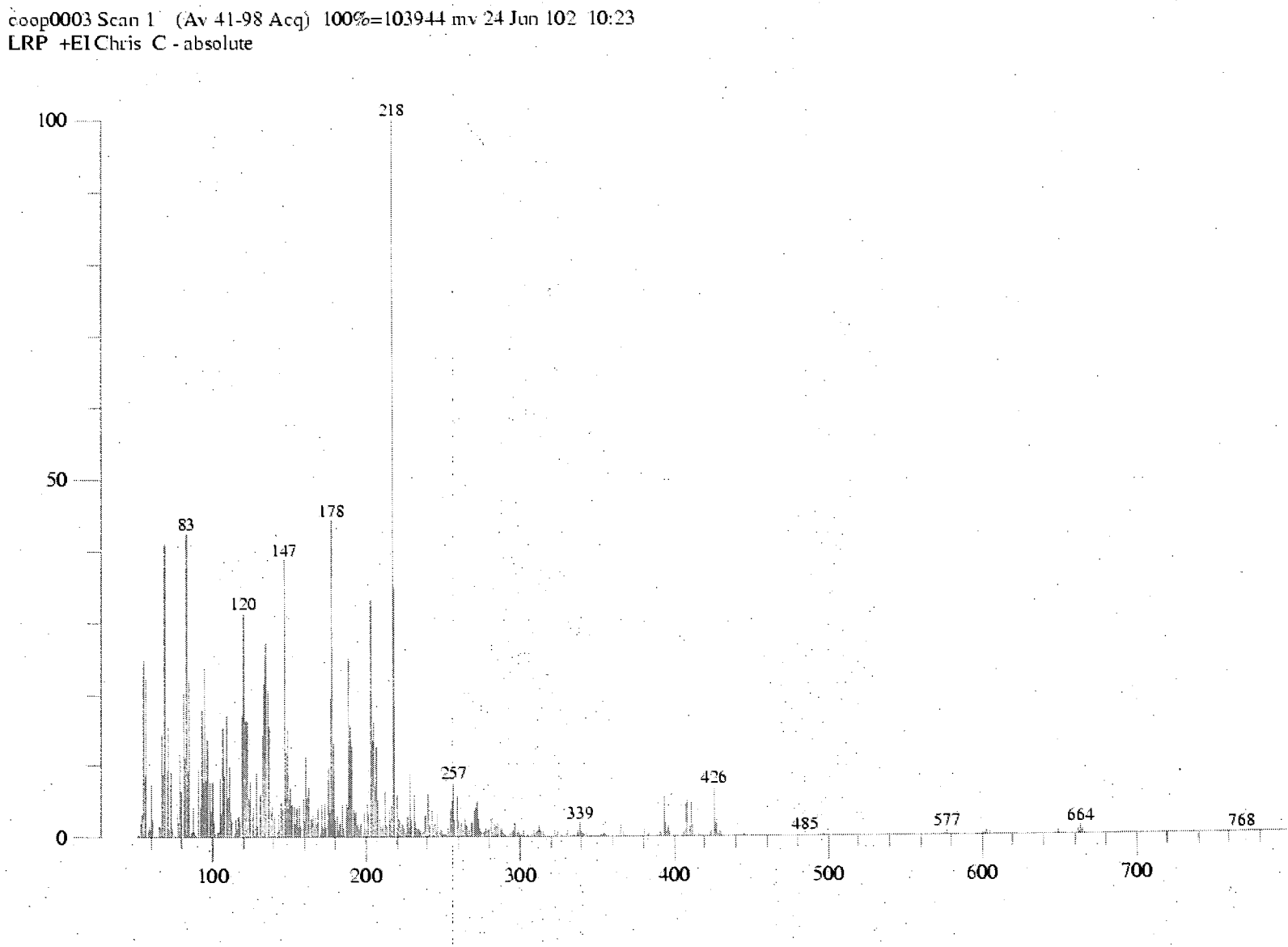




Appendix 3 - Temperature profile of commercial trial over the 4 day incubation period

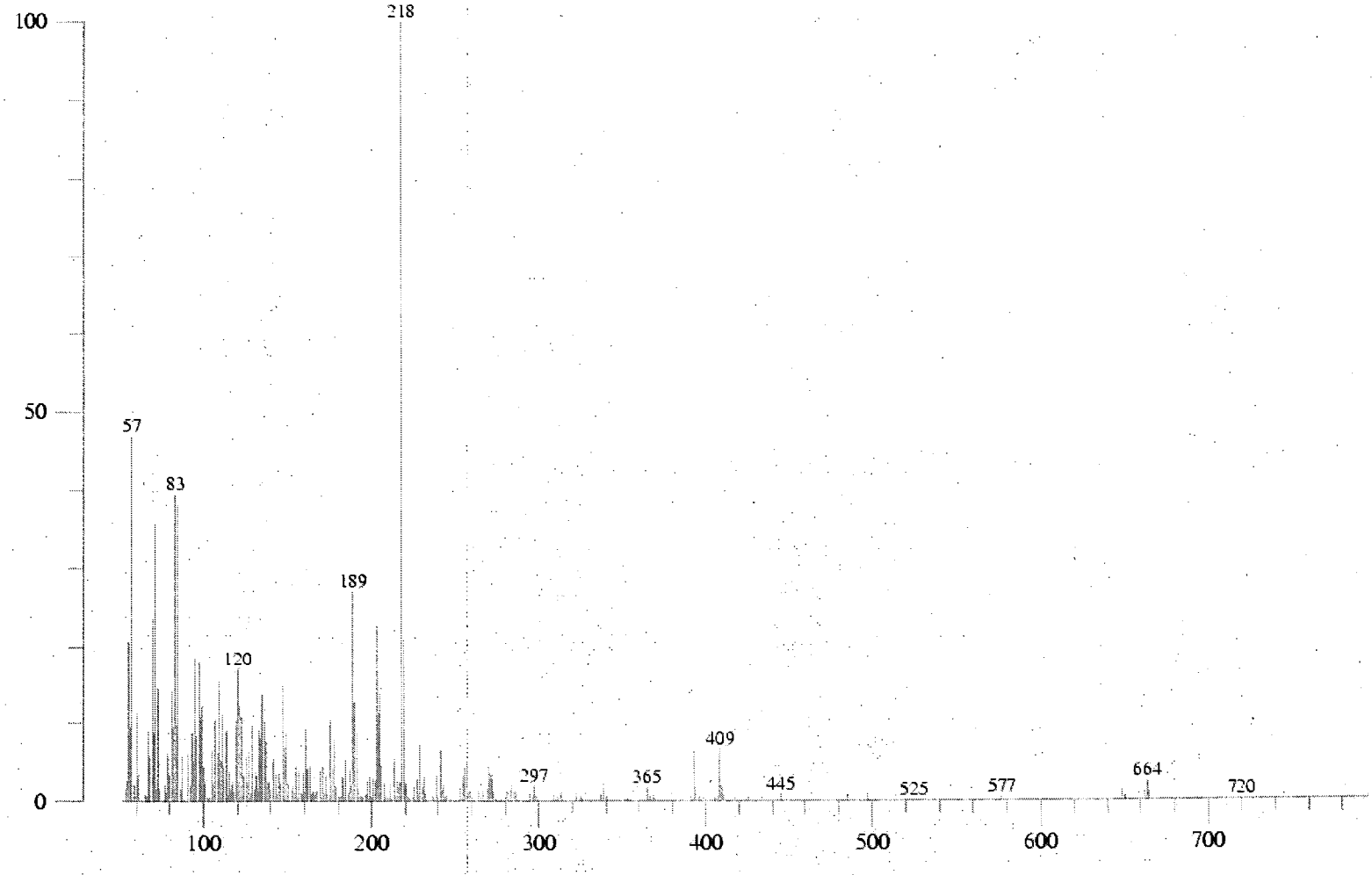


Appendix 4 A. Direct Probe Mass Spectrometry scan of absolute from fresh boronia flowers.



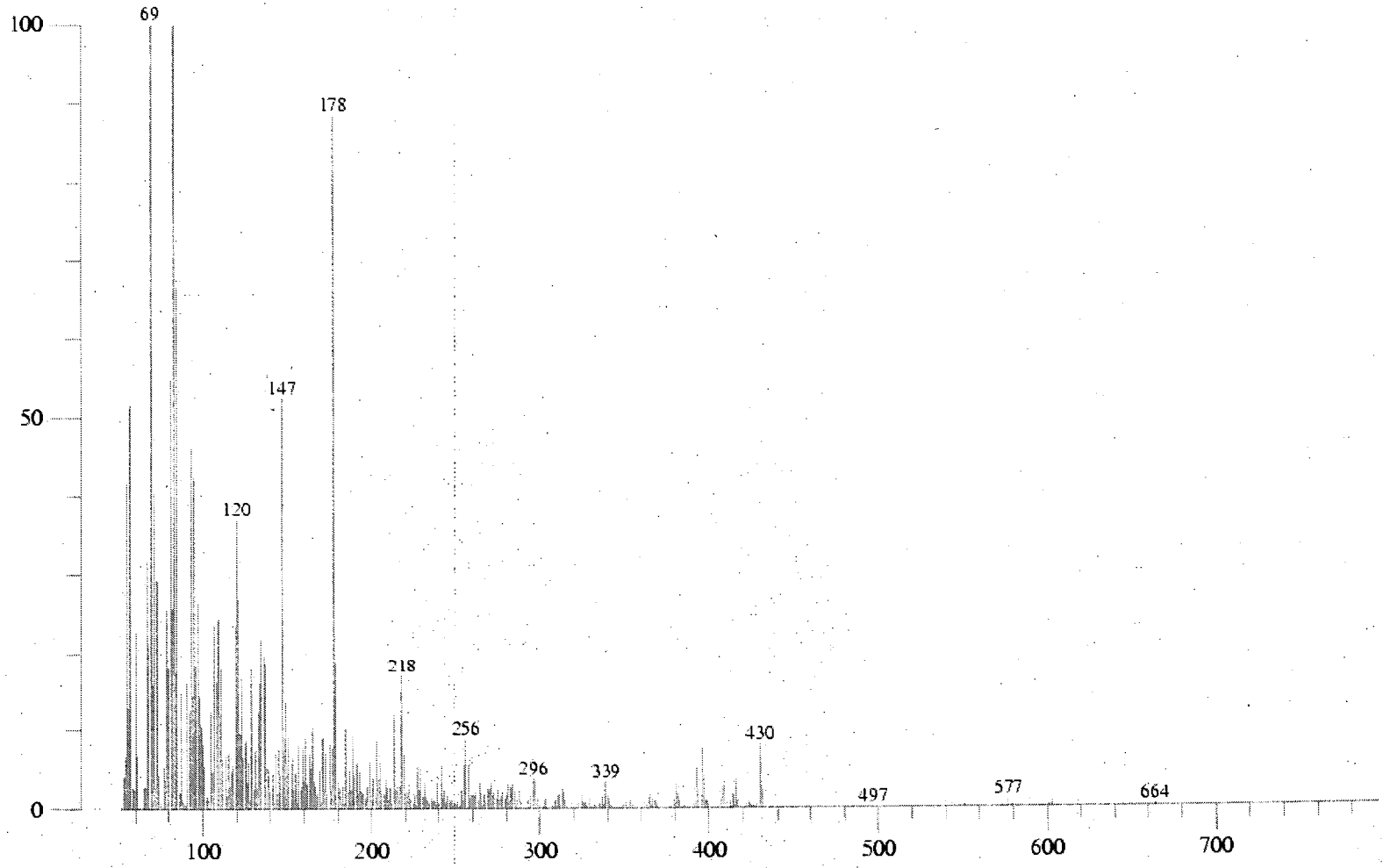
Appendix 4 B. Direct Probe Mass Spectrometry scan of concrete from fresh boronia flowers.

coop0008 Scan 2 (Av 93-160 Acq) 100%=47148 mv 24 Jun 102 13:21
LRP +EI Chris C - concrete

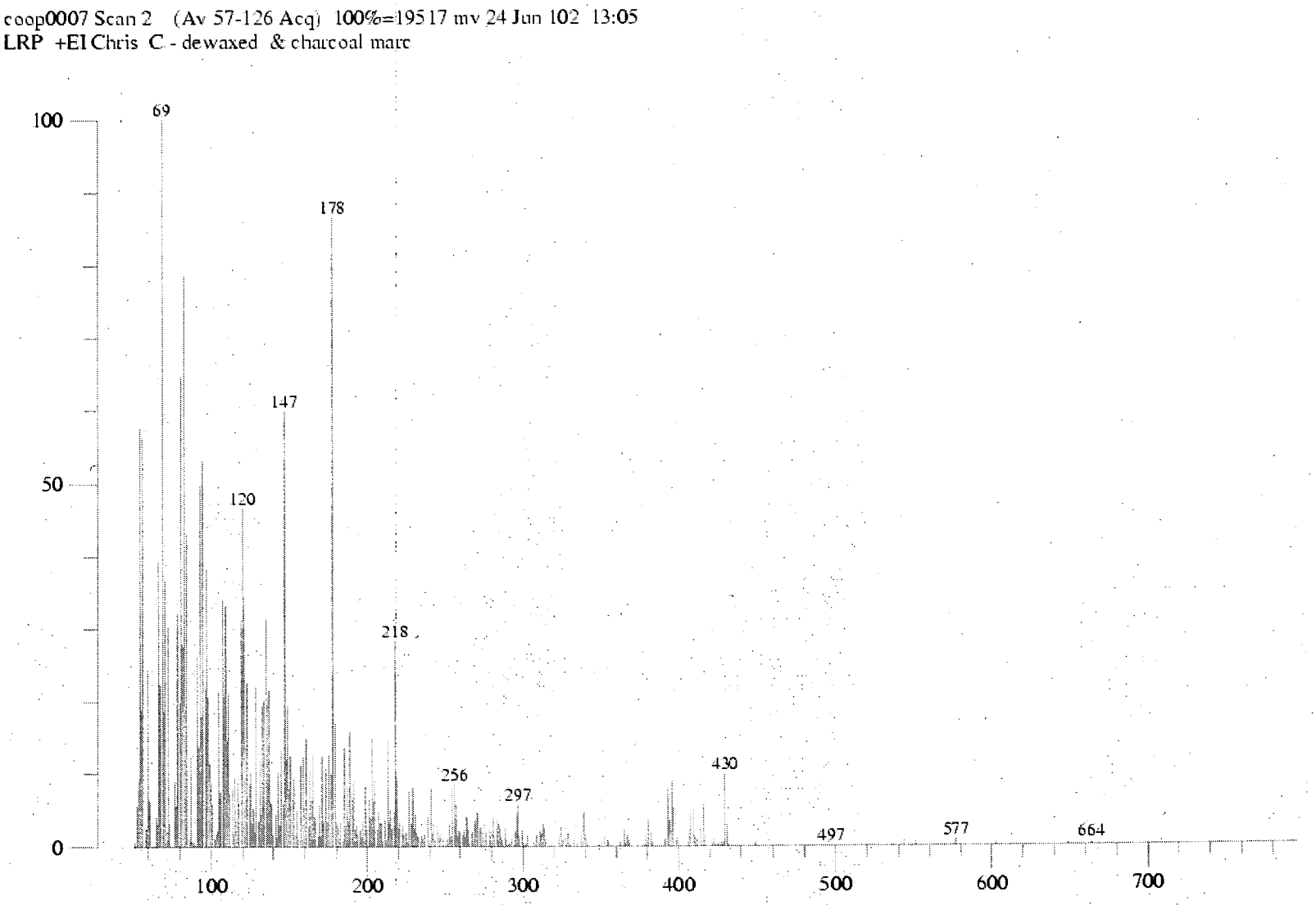


Appendix 4 C. Direct Probe Mass Spectrometry scan of a concrete from boronia marc.

coop0004 Scan 1 (Av 46-82 Acq) 100%=70252 mv 24 Jun 102 10:43
LRP +EI Chris C - marc



Appendix 4 D. Direct Probe Mass Spectrometry scan of an absolute (dewaxed and charcoal treated) from boronia marc.



Appendix 5 – Raw data for organoleptic assessment of four-day experiment.

Appendix 5 A. Organoleptic assessment of pottles from four-day fungal isolate trials.

		Organoleptic testing of pottles after 4 days
Sample No.	**Sample ID**	**Assessor's comments**
1	control	Predominately citrus, tobacco, cinnamon - balanced.
2		As above but stronger in tobacco
3		Tobacco and citrus slightly in favour of tobacco
4		Similar to 1 in balance
5	fungus 9	Very similar to 7
6		Mouldy and cinnamon - similar to 10
7		Strong tobacco / strong boronia flower smell - again slightly mouldy
8		Strong tobacco and cinnamon - slightly less of floral character
9	fungus 7a	Slightly musky with cinnamon and tobacco
10		Spicy and cinnamon
11		Good tobacco and citrus / floral like 7.
12		Strong citrus and slightly mouldy. Less of boronia (some tobacco)
13	fungus 2a	Quite strong floral and citrus / cinammon and resin
14		Defintely floral and citrus - slight tobacco and cinammon
15		Beautiful floral character - a bit like fressias and mandarins
16		Strong floral / resinous - very pleasant. Some citrus
17	fungus 8	Strong resin and; slight floral and citrus. Overipe mandarin smell
18		Strong resin and some floral, cinammon and tobacco.
19		Freesias and mandarin - not so much of the mouldy smell
20		Similar to 19, floral and citrus. Stronger resinous smell. Slightly off odour of sulphur.

Appendix 5 B. Organoleptic assessment of dry down flasks from four-day fungal isolate trials.

		Organoleptic testing of dry down flasks for 4 day experiment
Sample No.	**Sample ID**	**Assessor's comments**
1	control	Some characteristics of boronia concrete. Predominately hay with floral notes in the background.
2		Like boronia concrete with fruit. Bit of hay and tobacco but fruit is a bit stronger. The fruit is tending towards floral.
3		Different to the others in this group and a bit sharp. The first impact is almost acidic. Settles down to floral fruit and some hay.
4		Hay, tobacco and fruity undertones.
5	fundus 9	Similar to 6 and 8. A musky / dusty shoe polish type smell. Also spicy.
6		The first impression was tea - then leather boot polish. Then is greenish (finest and fresh leaves). A very volatile sense - good impact. Deep down is must and mouldy.
7		Less dusty and more fresh than 6. Fresh fruity notes. Green and herby.
8		Similar to 6. Also dusty with a musky element.
9	fungus 7a	Similar to 11
10		Very different. Initial smell like a cow manure. This settle down to fermenting hay with a bit of fruit in the background. Maybe a bit of sulphur given the cowyard smell.
11		Strong shoe polish. Settles down to new mown hay with a nice background of fruit.
12		Herbaceous. Smells like mown grass (Hexanol and hexanal). Some fruit present.
13	fungus 2a	Spicy note is predominating (close to cinnamon) with a slight tobacco smell. Nice fruit (not quite the persimmon in 14) more like a nutty smell.
14		Cinnamon and fruit which is a mixture of persimmon and another tropical fruit (perhaps guava).
15		Similar to 16 with a little more an interesting spice aroma - touch of cinnamon.
16		Tobacco that levels out and spice. Possibly fruit (persimmon).
17	fungus 8	A basic undertone of fruit and floral with hay over the top. Not as voluptuous of as 18 - 20. Some leafy herb (possibly coriander)
18		Some similarities to 20. Freesias, tropical fruit and citrus (lime).
19		Lacks the strong freesia smell. More of a herbaceous character with a background of floral. Some apricot with other tropical fruits like *Monsteria deliciosa*.
20		Freesias - lovely smell. Quite floral and sweet with some hay, violet and apricot.

Appendix 5 C. Organoleptic assessment from dilution analysis of four day fungal isolate trials.

		Dilution analysis - 4 drops
Sample No.	**Sample ID**	**Assessor's comments**
1	control	Citrus with some tropical fruit
2		Similar but stronger than 1
3		Similar to 1 with some hay and a little spice
4		First impression of soapiness. Otherwise fresh fruit with a little soapiness.
5	fungus 9	Fruit and some floral.
6		Spicey, - almost a touch tobacco. Also fruit - not as much spice as 9.
7		Spice predominates plus rounded fruit note inlcuding a little bit if citrus.
8		Similar to 7.
9	fungus 7a	Similar to 7 and 8.
10		Strong tropical fruits and some sub-tropical fruits.
11		First impact is a harsh note of shoe polish. Mixture of floral and fruit.
12		Floral. Heavy rose - quite strong.
13	fungus 2a	Fruit and slight floral.
14		A bit floral.
15		Similar to 13. Floral and fruit - jasmine and freesias.
16		Pungency of herb. A bit of cinammon - slight floral.
17	fungus 8	A bit hoppy. The freshness of lime
18		Violet smell. Sweet with top notes of boronia. Mixture of violet and freesias.
19		Spearmint with slight floral and hay.
20		Top notes of boronia plus a pleasant floral note and some apricot.

Appendix 5 D. Organoleptic assessment of tapers from four-day fungal isolate trials.

		Tapers
Sample No.	**Sample ID**	**Assessor's comments**
1	control	Fruit (tropical) lovely hay and a bit of floral.
2		Similar to 1 with strong hay.
3		Similar to 2 but with less hay.
4		Hay, tobacco and floral. A little lime and musk.
5	fungus 9	Lime.
6		Hay and floral
7		Some lime with a some fresh tropical fruit.
8		Floral and lime.
9	fungus 7a	Strong lime.
10		Mixture of lime and freesias.
11		Strong freesias and rose.
12		Jasmine and rose - quite strong.
13	fungus 2a	Similar to 12 but with pleasant and balanced floral elements.
14		Similar to 13.
15		Jasmine predominates. Contains other floral elements like rose but no sweetness.
16		A fermented character with a nice background of violet, hay and jasmine.
17	fungus 8	Spicy and slightly floral.
18		Similar to 17 with cinnamon and nutmeg.
19		Cinnamon and nutmeg with a little hop.
20		Fermenting - brewery smell.